

Studies in Chronic Allograft Nephropathy

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Acknowledgements and Declaration

I wish to acknowledge the important contribution of several individuals to the research work that is presented in this thesis. The original idea of performing the main study was conceived by senior transplant surgeons, physicians and clinical scientists in Leeds, Liverpool and Manchester (notably Dr CG Newstead, Mr AQ Hammad and Dr PEC Brenchley). I was involved personally in some of the discussions that led to refinements of the study protocol prior to its final approval. Patients receiving follow-up care at hospitals in West Yorkshire (St James's University Hospital in Leeds and Bradford St Luke's Hospital) and Liverpool (Royal Liverpool and Broadgreen University Hospitals) participated in the study. Laboratory work was performed in conjunction with Dr PEC Brenchley in the Renal Research Laboratories at the Manchester Institute of Nephrology and Transplantation. Dr CG Newstead and I were responsible for obtaining Local Research Ethics Committee approval for the study in Leeds. Funding for the work in Leeds was provided by the Yorkshire Kidney Research Fund as a result of approaches made by Dr CG Newstead and (more latterly) myself. Dr AF Mooney and I, in conjunction with Dr CG Newstead, prepared and submitted applications for additional funding from the Northern and Yorkshire Regional Research and Development Committee. The pharmaceutical companies Fujisawa and Roche funded the laboratory research in Manchester. I was responsible for the planning and implementation of the study in Leeds and Bradford except for obtaining ARSAC approval for the radioisotope studies (this was pursued by Dr CG Newstead and Dr MT Burniston from the Department of Medical Physics). The medical consultant staff in Bradford (notably Dr RF Jeffrey and Dr RG Roberts) provided helpful support. My responsibilities included patient identification, investigation (including a new arrangement with the Day Unit at St James's to perform renal transplant biopsies) and recruitment to the study. I conducted all review visits with the assistance of a research nurse (initially Sister H Ingles and latterly Mr J Carr). The research nurse was responsible primarily for visit scheduling and organisation of patient transport, and helped with collection and storage of blood and urine samples. I personally transferred samples to the Renal

Research Laboratories in Manchester. I received instruction in all of the relevant laboratory techniques and took responsibility for the entire process of analysing study samples. I am grateful to members of the laboratory staff (notably Mr ID Read, Dr S Williams, Dr BM Coupes and Dr PEC Brenchley) for their support and encouragement. I was responsible for data collection in Leeds and Manchester. Data from the Liverpool unit were collected by Mr G Owens and Mr M Paraoan and then forwarded to me for analysis. I had extensive discussions with a statistician from the University of Leeds (Dr AJ Baczkowski) who performed a more detailed independent analysis of the study data. *In vitro* cyclosporin sensitivity testing was performed in the Department of Transplant and Cellular Immunology at St James's Hospital by Dr B Clark and his staff. I was responsible for the collection and delivery of samples, aspects of data analysis and interpretation of the study findings. Pharmacokinetic profiling of cyclosporin was an initiative that I explored with Dr CJ Bowmer in the Department of Biomedical Sciences at Leeds University. I pursued the urine NAG and cystatin C work with Dr M Bosomworth in the Department of Chemical Pathology at St James's University Hospital. He and his staff were responsible for performing the relevant assays.

I hereby declare that I have been responsible for composing the thesis entitled 'Studies in Chronic Allograft Nephropathy'. The content of the thesis has been reviewed periodically by Dr CG Newstead, my project supervisor, and Dr R Baker. I have not submitted the thesis in candidature for any other degree, diploma or professional qualification.

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Abbreviations

ACE – angiotensin converting enzyme
ADPKD – autosomal dominant polycystic kidney disease
Ang II – angiotensin II
AR – acute transplant rejection
AT1-RA – angiotensin II receptor antagonist
AUC – area under the time-blood CsA concentration curve
AZA – azathioprine
bp – base pairs
C2 – the 2 hours post-dose blood cyclosporin level
CAN – chronic allograft nephropathy
CMV – cytomegalovirus
CRAD – chronic renal allograft dysfunction
CsA – cyclosporin
CvarCo – the cyclosporin trough level variation coefficient
DNA – deoxyribosenucleic acid
EDTA – ethylenediamine tetra-acetic acid
ELISA – enzyme-linked immunosorbent assay
ESRF – end-stage renal failure
EU – European Union
FK506 – tacrolimus
FVL – factor V Leiden
GBM – glomerular basement membrane
GFR – glomerular filtration rate
HBA1C – glycosylated haemoglobin
HBV – hepatitis B virus
HCV – hepatitis C virus
HD – haemodialysis
HIV – human immunodeficiency virus
HLA – human leucocyte antigen

HMG-CoA reductase – hydroxy-methylglutaryl coenzyme A reductase
IQR – interquartile range
ITN – Immune Tolerance Network
LR – lymphocyte responsiveness
MHC – major histocompatibility complex
MLC – mixed lymphocyte culture
MMF – mycophenolate mofetil
MTHFR – methyltetrahydrofolate reductase
NAG – N-acetyl β -D glucosaminidase
NIBSAC – National Institute of Biostandards and Controls
pmp – patients per million population
RFLP – restriction fragment length polymorphism
RPM – rapamycin
PCI – protein creatinine index
PCR – polymerase chain reaction
PD – peritoneal dialysis
PENIA – particle-enhanced immunonephelometric assay
PETIA – particle-enhanced turbidometric assay
PHA – phytohaemagglutinin
RFLP – restriction fragment length polymorphism
ROC – receiver operating characteristics
ROCT – reciprocal of creatinine versus time
RPM - rapamycin
RTR – renal transplant recipient
SD – standard deviation
SRID – single radial immunodiffusion
TGF-beta – transforming growth factor beta
TNF-alpha – tumour necrosis factor-alpha
UNOS – United Network for Organ Sharing
UK – United Kingdom
USRDS – United States Renal Data System

Abstract

Background and Methods

Following renal transplantation there is excellent 1-year graft survival but thereafter a steady rate of graft loss that is due mainly to premature patient death or cumulative graft injury as occurs in chronic allograft nephropathy (CAN). CAN is a clinico-pathological term that applies to renal transplants when there is a decline in function in association with fibrointimal hyperplasia of blood vessels and progressive scarring of the graft interstitium. There are both immunological and non-immunological aetiologies of CAN. Renal transplant recipients with allograft failure secondary to CAN account for roughly 3% of all entrants to chronic dialysis programmes in the UK (approximately 150 per annum), increasing the demand placed on this limited resource. Allograft failure also has a considerable impact on patients awaiting call-up for transplantation, for example as many as 1 in every 6 patients transplanted in the UK in the year 2000 had previously received a renal transplant. The number of patients listed for renal transplantation in the UK has increased by more than 50% since 1990 and currently exceeds 6,000. The clinical management of renal transplant recipients (RTRs) should therefore include anticipation, prevention, early detection and optimal treatment of CAN. The component studies of my thesis have addressed specific areas within each of these main divisions. They can be summarised as follows: *screening tests* for potential RTRs that may establish a predisposition to the development of CAN within their allograft (*genetic profiling* for Transforming Growth Factor-beta 1 (TGF-beta 1), a cytokine that promotes arteriosclerosis and

fibrosis, and *lymphocyte sensitivity* to the immunomodulatory effects of CsA *in vitro* using a mitogenic stimulus); measurement of the degree and variability of *CsA exposure* (based on C2 and C0 values); evaluation of candidate *urine markers for incipient CAN* (TGF-beta 1 and the lysosomal enzyme NAG); validation of alternative *measurements of renal transplant function* that are more sensitive to small reductions in glomerular filtration rate (serum cystatin C and creatinine-based GFR formulae); and finally a prospective, randomised controlled study of *immunosuppression conversion for patients with established CAN* (MMF and reduced dose CsA vs tacrolimus for CsA vs 'no change' controls).

Results and discussion

The notion of a screening programme for potential RTRs to reduce the prevalence of CAN through identification of those 'at risk' is a credible one. However, the inclusion in such a programme of genetic profiling to predict post-transplant TGF-beta 1 expression and measurement of lymphocyte sensitivity to CsA *in vitro* is not supported by the study data. Modified studies that examine larger populations and combinations of screening tests may be contemplated in the future. Control groups need to be rigorously defined, for example by inclusion of histopathological criteria.

It would also appear that the measurement of candidate urine markers for *incipient CAN* (TGF-beta 1 and the lysosomal enzyme NAG) is of little predictive or diagnostic value. Proteomic analyses may help to identify a family of discriminatory urine markers for inclusion in future non-invasive graft surveillance programmes.

The studies relating to measurement of serum cystatin C (a more sensitive marker of small reductions in GFR than serum creatinine) and calculation of GFR using the

Levey (MDRD) prediction formula indicate that both are useful in the detection and/or quantification of early renal allograft dysfunction. A greater awareness of diminishing renal clearance would increase the opportunity for effective therapeutic intervention.

The studies of blood CsA monitoring indicate that a more accurate quantification of CsA exposure may be important in reducing both the incidence of acute transplant rejection during the immediate post-transplant period (a well documented risk factor for the development of CAN) and the rate of chronic disease progression in established CAN. A large intra-individual variation in CsA exposure may indicate poor adherence to treatment regimens rather than variation in drug bioavailability.

In the study of immunosuppression conversion for RTRs with established CAN, a comparison of reciprocal of creatinine vs time (ROCT) slopes before (-12/12 – 0) and after (0 – 6/12) intervention revealed a treatment advantage for patients whose immunosuppression was changed to MMF/ reduced dose CsA ($p < 0.05$). Similar results were obtained in the GFR analysis. Exclusion of patients with an initial GFR $< 20 \text{ ml/min/1.73m}^2$ from the analysis gave a result that was statistically and clinically more striking ($n = 27$, $p < 0.05$), indicating that immunosuppression conversion should be considered at an early stage in the development of CAN. A comparison of pre-study and 3/12 – 12/12 ROCT slopes (to isolate the early effect of CsA dose reduction on renal transplant haemodynamics) showed a non-significant difference between treatment groups (MMF/ reduced dose CsA vs controls, $p = 0.08$), suggesting that the CsA dose reduction component of the regimen is most beneficial. The number of study participants was suboptimal, mainly because of slow recruitment in some of the participating centres. A more extended period of follow-

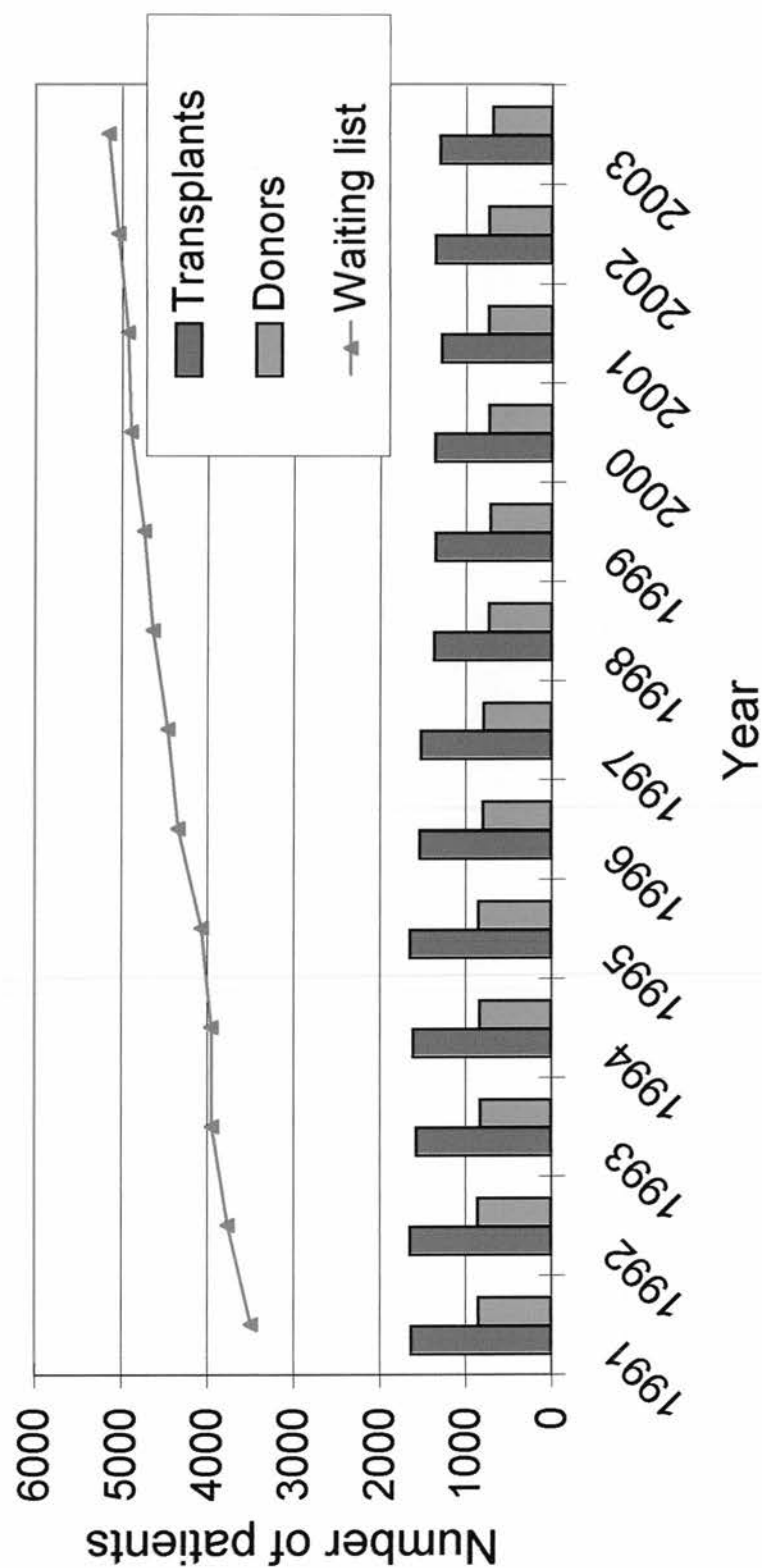
up would have provided information about the longer-term safety and effectiveness of MMF in combination with reduced dose CsA for the treatment of CAN.

Introduction

There is a major problem of donor organ shortage in the United Kingdom (UK). We have one of the lowest European donor rates at around 13.5 pmp per annum. Between 1990 and 1998, there was an 18% reduction in the annual number of cadaveric renal transplants in the UK and a 50% increase in the number of patients on the transplant waiting list (Figure 1). This trend has continued up to the present day. The widening gap between supply and demand inevitably results in a lengthening of the average waiting time for renal transplantation (UK Transplant 2001). The number of patients on either the 'active' or 'temporarily suspended' UK renal transplant waiting lists at the end of December 2003 was 6,447, a rise of 2% on the previous year's total. The situation would be even worse if it were not for a recent expansion of living donor programmes across the UK. In the year 2003, 439 living donor renal transplants were performed, accounting for just over a quarter of all UK renal transplant activity.

What can be done to tackle the problem? There are a number of challenges facing the transplant community. It is essential that the general public is informed about organ donation, and individuals must be encouraged both to join the Organ Donor Register and to discuss with family members their wish to be considered as potential organ

Figure 1. Cadaveric kidney programme in the UK 1991 - 2003
 Number of donors, transplants and patients on the active waiting
 list (source UK Transplant Activity Report 2000)



donors in the event of their death. A more dramatic step would be to change from an 'opt in' to an 'opt out' system of organ donation (whereby every person living in the UK would be deemed to have consented to organ donation unless they had previously registered their objection to the same). This approach raises a number of difficult ethical issues and opinion is understandably divided. The recent UK Potential Donor Audit has shown that approximately 50 % of families approached about organ donation are prepared to give their consent (UK Transplant 2004). The report also states that an increase in the number of UK transplant coordinators is necessary to maximise donation rates from both heart-beating and non-heart beating donors (especially within the non-white population). These ideas have been brought together in a blueprint for UK transplant services published by the Department of Health (Saving Lives, Valuing Donors – A Transplant Framework For England, Department of Health 2003). Other service priorities include improved HLA matching of cadaveric renal allografts (new arrangements for organ sharing within large alliances such as the North of England Transplant Alliance (NETA) have already made an important difference in this regard) and better characterisation of recipient anti-HLA antibodies. The latter will reduce the likelihood of an unexpected positive crossmatch test and therefore improve the efficiency of organ allocation. There is also interest in developing more effective organ preservation solutions that will reduce cold injury and the occurrence of delayed graft function.

The incidence of acute renal transplant rejection is much lower than in the past for a number of reasons, but the absence of a proportionate improvement in long-term transplant survival raises two other important challenges, namely a reduction in patient mortality (prevention of premature cardiovascular disease will be the key

aim) and graft loss resulting from *chronic allograft nephropathy* (CAN). Renal transplant recipients with allograft failure secondary to CAN account for roughly 3% of all entrants to chronic dialysis programmes in the UK (approximately 150 per annum), increasing the demand placed on this limited resource. Allograft failure also has a considerable impact on patients awaiting call-up for transplantation, for example as many as 1 in every 6 patients transplanted in the UK in the year 2000 had previously received a renal transplant.

In this thesis I have considered a number of approaches to the specific challenge of minimising the negative impact of CAN on renal transplant survival. The studies are wide-ranging and can be placed into three main categories (other well-established approaches are included in italics):

(1) Anticipation/ prevention of CAN - identification of predisposing factors

- *Organ quality (donor factors, procurement and preservation factors)*
- *HLA match*
- Cytokine genotype
- In vitro cyclosporin (CsA) sensitivity
- CsA exposure
 - C2 (the 2 hours post-dose blood CsA level) monitoring and the prevention of early acute transplant rejection (AR)
 - intraindividual variability of CsA exposure (CvarC0)

(2) Early non-invasive methods of detecting CAN

- Urine markers – TGF-beta (Transforming Growth Factor beta) / N-acetyl β -D glucosaminidase (NAG)

- Serum cystatin C
- GFR (glomerular filtration rate) prediction formulae

(3) Treatment of CAN

- *Blood pressure control/ lipid-lowering therapy/ ACE (angiotensin converting enzyme) inhibitor therapy*
- Randomised controlled trial of mycophenolate mofetil (MMF) and CsA dose reduction/ tacrolimus in place of CsA/ 'no change' standard dose CsA for patients with established CAN

The concepts underlying these studies are outlined in the opening sections of the relevant chapters. The final chapter contains a general summary of the study findings and future strategies that may help to prevent, identify and treat CAN.

End-stage renal failure (ESRF) – aetiology,
incidence, morbidity and mortality

2.1 Aetiology of ESRF

Progression to end-stage renal failure (ESRF) occurs in a large proportion of patients with chronic renal disorders. Diabetes mellitus and hypertension are the most common causes of ESRF in the United Kingdom (UK) followed by the various glomerulonephritides. Stability of renal function may be achieved in patients with hypertensive nephrosclerosis and obstructive uropathy, where the precipitating factor can be controlled to some extent by appropriate therapy. Progression to ESRF tends to occur more rapidly in chronic glomerular disease and diabetic nephropathy than in autosomal dominant polycystic kidney disease (ADPKD) and chronic tubulointerstitial disease. The rate of disease progression correlates positively with the degree of proteinuria (Williams *et al* 1988).

It is understood that the mechanism of progressive renal injury ('scarring') is common to nephropathies of diverse aetiologies and is influenced by genetic, haemodynamic, metabolic and dietary factors. The relevance of such factors to the pathogenesis of chronic allograft nephropathy (previously termed chronic transplant rejection) will be discussed in a later section. Control of blood pressure and moderation of dietary protein and saturated fat intake may slow the rate of disease

progression, irrespective of the underlying renal pathology (Levey *et al* 1996, El Nahas 1998).

2.2 Incidence of ESRF

The incidence of ESRF in different parts of the world varies with population demographics and the quality of local health care provision. The UK Renal Registry Report summarises data from more than 30 renal units in England, Scotland and Wales (Ansell *et al* 1999). In 1998, 92.2 new patients per million population (pmp) per year required renal replacement therapy, and the point prevalence of adult patients receiving renal replacement therapy in the whole of the UK was estimated to be 31,316 (more than 500 pmp). One year later, the number had increased by approximately 4.3%. This trend is likely to continue well into the future. Approximately 50% of prevalent patients are on haemodialysis (HD) or peritoneal dialysis (PD) and the remainder have a functioning renal transplant.

The most comprehensive epidemiological data for dialysis populations are provided by the United States Renal Data System (USRDS, University of Michigan). It is apparent that the number of patients receiving maintenance dialysis in the United States is increasing at a net rate of more than 10% per annum and currently exceeds 300,000 (more than 600 pmp). Similar rates of increase have been reported in Europe and parts of the UK (Berthoux *et al* 1999; Ansell *et al* 1999). In comparison with historical dialysis populations, new patients are generally older and more likely to be diabetic and/or have significant comorbidity (for example atherosclerotic coronary artery disease, cerebrovascular disease, peripheral vascular disease, chronic obstructive pulmonary disease or cancer).

2.3 Morbidity and mortality of patients with ESRF receiving renal replacement therapy

Dialysis treatment can offer good quality rehabilitation to patients with ESRF, but overall there is premature mortality. Risk factors for the development of atherosclerotic coronary artery disease (such as anaemia, hypertension and hypercholesterolaemia) are prevalent in pre-dialysis populations and are not influenced significantly by renal replacement therapy in the form of HD or PD.

The death rate from ESRF in the European Union (EU) during 1996 was 69 pmp per annum. The death rate in non-diabetic patients aged 45 to 54 years with ESRF was 5 to 10 times higher than in matched cohorts of the general population (Brunner *et al* 1992). The annual mortality rate (number of deaths/ patient years at risk of death x 100) for ESRF patients on dialysis in the USA in 1997 was 20% (USRDS Annual Data Report 1998). The corresponding figure for UK patients was 26.3% (Renal Registry data). The apparent difference in mortality between the two countries may be explained by differences in methods of calculation (deaths that occur within the first 90 days of commencing chronic dialysis therapy are discounted in USRDS analyses). Cardiovascular disease is the most common cause of death for ESRF patients, followed in frequency by infection (Valderrabano *et al* 1993).

Death from cardiovascular disease occurs in *renal transplant recipients* at approximately half the overall rate for renal failure patients. The difference is due in part to selection of only the fittest patients for transplant listing (Briggs *et al* 1998). Patient and graft survival rates are discussed in Chapter 3. The broad picture is one of excellent one-year graft survival but thereafter a steady rate of graft loss that is consequent upon either premature patient death (patient survival at 5 years after renal

transplantation is 85-90%) or cumulative graft injury (such as occurs in chronic allograft nephropathy). It is argued by some that patient death is an acceptable form of graft loss in that the transplant has successfully provided life-long renal replacement. The key issue is whether or not patients should be screened thoroughly prior to transplantation and then excluded from transplant listing if their overall prognosis is poor, thereby making more efficient use of a limited resource. Decisions to exclude patients in this manner are of course complex and controversial and there are likely to be inconsistencies of approach between and within renal units.

Turning briefly to quality of life issues, subjective quality of life estimations and employment rates are significantly higher for renal transplant recipients (especially those who receive a kidney from a living donor) than for patients who remain on chronic dialysis treatment. This reflects the clear superiority of transplantation in terms of renal clearance and consequently the removal of restrictions on diet, fluid intake, working patterns, travel and so forth. There are particular benefits of transplantation in terms of improvement in anaemia, peripheral and autonomic neuropathy and sexual function.

Patient and graft survival following renal transplantation

Patient death accounts for between 22% and 48% of renal allograft loss after the first post-transplant year depending on the age and comorbidity of the recipient population (Bia 1995). The incidence of myocardial infarction and cerebrovascular events may be 25-fold higher than in the general population (Meyer *et al* 1996, Kasiske *et al* 2001). Atherosclerotic deaths account for up to 55% of all late transplant deaths in some series (Meyer *et al* 1996). Infection and malignancy are more common in immunosuppressed patients and account for approximately a third of late deaths. Less common causes of death include hepatobiliary disease and suicide. The incidence of suicide is 1% in some series, which is 50 times higher than the corresponding figure for age-matched controls.

Short-term *graft survival* in the UK has increased steadily in recent years due in part to more effective immunosuppressive therapy. The one-year survival rate for first cadaveric transplants performed in 1997 and 1998 (defining graft loss as a return to chronic haemodialysis or death of the recipient) was 86% (UK Transplant 2001). In the USA, the monthly rate of cadaveric renal allograft loss during the first 3 months post-transplant fell from 9.7% to 3.8% between 1983 and 1992 (USRDS Annual

Report 1995). The attrition rate in subsequent months was also reduced, from 1.8% to 0.6% for the 4–12 months post-transplant period, and from 1.0% to 0.5% during the second post-transplant year. The projected half-life of primary cadaveric grafts (the estimated number of post-transplant years at which only one half of first transplants will be functional) transplanted in 1995-96 is 100 months. This statistic compares favourably with a projected half-life of 80 months for grafts transplanted in the early 1990s. Hariharan *et al* recently concluded that improvements in graft half-life are related to conservation of renal function in the first year post-transplantation (Hariharan *et al* 2002). The graft half-life estimates for *living donor* grafts transplanted in 1990-91 and 1995-96 are 130 months and 175 months respectively.

However there is some cause for concern. According to USRDS data, there was no change in the monthly rate of cadaveric allograft loss after the second post-transplant year (0.7% versus 0.8%) between 1983 and 1988. Similarly, the 5-year graft survival rate in the UK has remained disappointingly low at around 65%. It is likely that both immunological and non-immunological factors determine graft outcome. These are discussed in Section 4.1.

Renal transplants fail for many reasons (Thiru 2001). A small number fail immediately after transplantation for technical reasons. The rare complication of *arterial and/or venous thrombosis* of the graft occurs typically within the first 2-3 days post-transplant but may occur much later as a complication of an acute rejection episode. Problems relating to the arterial anastomosis are more likely if the donor kidney is supplied by more than one renal artery. Thrombosis is heralded by a sudden cessation of urine output and, in the case of venous occlusion, by graft swelling and localised pain. The majority of grafts in which arterial or venous thrombosis occurs

have irrevocable loss of function. Early *ureteric* complications include urinary leak following anastomotic breakdown and obstruction resulting from kinking, stenosis or stent blockage.

Early graft loss as a consequence of *accelerated rejection* is an extremely rare event. It is triggered by the action of pre-formed recipient cytotoxic antibodies that are directed against either human leucocyte antigens (HLAs, see Section 4.1) or ABO blood group antigens expressed by the donor. It occurs immediately post-transplantation (*hyperacute rejection*) although *delayed hyperacute rejection* occurring several days after engraftment is also recognised. Cytotoxic alloantibodies bind to the endothelial surfaces of graft arterioles and activate complement, leading to severe vascular injury with arteriolar occlusion and thrombosis. Patients are usually oligoanuric and have a fever and graft tenderness. It is often necessary to perform a transplant nephrectomy. A pre-transplant crossmatch test (incubation of donor lymphocytes with recipient serum in the presence of complement, allowing detection of donor-specific cytotoxic antibody) is performed routinely to reduce the incidence of adverse clinical outcomes resulting from such donor-recipient incompatibility.

Acute rejection may also result in graft loss. *Cell-mediated acute rejection* is the most common form of acute rejection. Donor antigens, particularly HLA antigens, are recognised by specific T cells that induce either direct cytotoxicity or a delayed hypersensitivity response. The major histopathological feature is a widespread infiltration of the renal interstitium by leucocytes, most of which are mature T cells. Lymphocytes and monocytes also extend into the walls and lumina of renal tubules ('tubulitis') and the vascular endothelium. *Antibody-mediated acute rejection* is less

common and is characterised primarily by necrotising arteritis with fibrinoid necrosis and a neutrophilic infiltrate. Endothelial cells are severely damaged or absent and luminal thrombosis is common. There may be infarction of the renal cortex with interstitial haemorrhage. Immunofluorescence studies typically reveal deposition of IgG and C3 in the walls of arteries. The introduction of more potent immunosuppressive agents and improvements in tissue typing in recent years has reduced greatly the risk of catastrophic acute cellular or vascular rejection.

Chronic renal allograft dysfunction (CRAD) may result from a variety of processes but is often secondary to *chronic allograft nephropathy* (CAN, see below). Estimation of the true prevalence of CAN would require regular 'protocol' biopsies of all transplant recipients even when graft dysfunction is not evident, a policy that is adopted in only a few centres. Solez *et al* found histopathological evidence of CAN at 2 years post-transplant in approximately two-thirds of cadaveric renal transplants receiving calcineurin-inhibitor immunosuppression (Solez *et al* 1998). Serum creatinine is used as a marker of glomerular filtration rate (GFR) in clinical practice but it is insensitive to changes in renal function when the GFR is more than 75ml/min/1.73m² (Stewart Cameron *et al* 1998). There is a hyperbolic relationship between GFR and serum creatinine, such that GFR values in excess of 75ml/min/1.73m² are represented on a relatively flat portion of the 'serum creatinine vs GFR' curve and any decrease in GFR will produce only a small increase in serum creatinine. Conversely, a decrease in GFR for patients with pre-existing renal impairment (for example a GFR of less than 30ml/min/1.73m²) is associated with a relatively large increase in serum creatinine (i.e. serum creatinine is a more sensitive marker of change in renal function within this GFR range). Another source of

difficulty in estimating the prevalence of CAN is differentiating between the histopathological features of CAN and those of other processes such as ischaemic nephropathy and cyclosporin (CsA) toxicity. This is discussed in more detail in Section 4.5.

Chronic allograft nephropathy (CAN)

Chronic allograft nephropathy (CAN) is a poorly characterised clinico-pathological term that applies to renal transplants when there is a slow and often variable rate of decline in function with fibrointimal hyperplasia of blood vessels, glomerular changes and progressive scarring of the graft interstitium. Urinary protein excretion exceeds 0.5 g/day in approximately a quarter of cases (Massy *et al* 1996). A diagnosis of CAN requires exclusion of other causes of graft dysfunction such as hypoperfusion, urinary tract obstruction, recurrence of original renal disease, allergic interstitial nephritis, viral infection (cytomegalovirus and BK polyoma virus), hypertensive nephropathy and drug nephrotoxicity (including the calcineurin inhibitors cyclosporin (CsA) and tacrolimus (FK506)), although a multifactorial aetiology is seen in many patients. Investigation may include an ultrasound scan of the transplant, estimation of renal blood flow using colour-flow Doppler and a transplant biopsy.

CAN is the most frequent cause of late allograft failure excepting death with a functioning transplant. United Network for Organ Sharing (UNOS) Scientific Renal Transplant Registry data for a cohort of *de novo* cadaveric renal allograft recipients transplanted in 1994–95 have shown that 9% of graft loss in the first year resulted from chronic rejection, 24% from acute rejection and 28% from death with a

functioning transplant. In the second post-transplant year, 34% of graft loss was attributed to chronic rejection, 6% to acute rejection, and 38% to patient death (Cecka 1996). It has been estimated that as many as 40% of patients with normal or near-normal transplant function will have mild histological features of CAN on biopsy (Mahony *et al* 1987; Dennis *et al* 1989; Insoniemi *et al* 1992). Progression to ESRF may occur in a few months or over a period of several years (Kasiske *et al* 1991).

UK transplant recipients with allograft failure secondary to CAN account for roughly 3% of all entrants to chronic dialysis programmes (approximately 150 per annum), increasing the demand placed on this limited resource. Allograft failure also has a considerable impact on patients awaiting call-up for transplantation, for example as many as 1 in every 6 patients transplanted in the UK in the year 2000 had previously received a renal transplant.

Renal transplantation is of course the most cost-effective way of managing patients with ESRF (United States Renal Data System Annual Report 1993). The cost per annum for a patient receiving dialysis treatment in the UK in 1992 was calculated to be £21,000, compared to £4,000 per annum for a renal transplant recipient (Mallick 1997). The annual net cost to the NHS for treating patients who return to dialysis because of graft failure is therefore likely to be in the order of hundreds of thousands of pounds (Morris-Stiff *et al* 1998a).

4.1 Aetiology of CAN

It is probable that there are multiple aetiologies of CAN, both *immunological* (HLA mismatch (Kupin *et al* 1997), frequency and severity of acute rejection episodes (Van Saase *et al* 1995; Matas *et al* 1994), CsA dose (Almond *et al* 1993)) and *non-immunological* (warm and cold ischaemia time (Shoskes *et al* 1998; Yilmaz *et al* 1993), donor age (Terasaki *et al* 1992), size mismatch of donor and recipient (Kupin *et al* 1997), hypertension (Opelz *et al* 1998), hypercholesterolaemia (Brazy *et al* 1992), viral infection (Lemstrom *et al* 1994)), that link into a final common pathway of fibrosis or scarring. A change in the nomenclature from chronic rejection (implying a purely immunological aetiology) to CAN lends greater emphasis to this concept.

Immunological factors

The slower rate of chronic transplant loss in well-matched recipients compared to poorly-matched recipients may be taken as a measure of the contribution of immunological factors to the development of CAN, although there are studies in which a survival advantage has not been demonstrated (Kerman *et al* 1993; Reinsmoen 2000). The latter may be related to a narrow distribution of human leucocyte antigen (HLA, see below) matching within some transplant populations, making it more difficult to demonstrate a beneficial effect of matching on outcome. It may be also that there are more appropriate ways of assessing histocompatibility than HLA match. For example, one recent study demonstrated that the number of cross-reactive epitope group (CREG) matches, but not the number of HLA mismatches, predicted long-term graft survival (Sijpkens *et al* 1999). The CREG

system represents a broader grouping of HLA epitopes (amino acid sequences within antigen binding sites) that are shared among different HLA specificities. Data from the Collaborative Transplant Study and our own population of renal transplant recipients suggest that CREG matching is of little benefit in terms of long-term transplant function (Wujciak *et al* 1999, Clark *et al* 2002). The average half-life of cadaveric grafts where there is a complete donor-recipient mismatch at the HLA A, B and DR loci has been estimated to be 7.4 years, compared to a half-life of 16.5 years for grafts that are perfectly matched at these loci (Terasaki *et al* 1990). In the UK there have been significant improvements in HLA matching following a revision of the rules governing the National Kidney Allocation Scheme.

MHC (major histocompatibility complex) antigens play a central role in the immune response to transplanted foreign tissue. The genetic code for these antigens is similar in all mammalian species. Characterisation of the human MHC (also referred to as the HLA system) has improved following the advent of DNA typing methods. MHC class I antigens (a composite group incorporating HLA categories A, B and C) are expressed on the surface of most nucleated cells and are responsible for the presentation of foreign antigen to CD8-positive T cells. T cells can be divided into two major subpopulations on the basis of their cell membrane glycoproteins (CD8 and CD4). It is thought that CD8 and CD4 are receptors for MHC class I and class II antigens respectively. MHC class II antigens (HLA category D) are expressed mainly on resting B cells, macrophages and some endothelial cells, although the cytokine tumour necrosis factor alpha (TNF- α) may induce expression on other cell types (e.g. renal tubular cells and T cells) during an episode of inflammation. The MHC demonstrates a remarkable degree of genetic polymorphism. Even within national

transplant organisations managing large pools of donors and recipients, avoidance of donor-recipient mismatch at the HLA A, B and DR loci (classified by serological typing methods) is achieved in only 10% of cases. The existence of 'permissible' and 'taboo' mismatches suggests that, in addition to 'public' determinants for given HLA specificities, there are 'private' determinants (amino acid sequences) that may or may not be homologous with the 'private' determinants of other HLA specificities (Dupont *et al* 1997). MHC incompatibility (particularly MHC class II) is the prime cause of acute allograft rejection (Basadonna *et al* 1993).

HLA-specific anti-lymphocyte antibodies are present in a large proportion of patients awaiting renal transplantation, mostly as a result of exposure to non-inherited maternal antigens (NIMAs) *in utero*, pregnancy, transfusion of blood products and previous engraftment. The degree of HLA sensitisation for an individual is expressed as %PRA ('panel reactive antibodies'). In simple terms, %PRA may be defined as the proportion of panel lymphocytes (taken from a random sample of individuals and therefore representative of the distribution of donor HLA phenotypes within the general population) that react to antibodies in patient serum. A minority of patients (10 - 20%) are 'highly sensitised', that is one or more of their stored serum samples (collected at regular intervals prior to transplantation) reacts with 80% or more of the panel lymphocytes (>80% PRA). According to data from the United Network of Organ Sharing (UNOS) Scientific Registry, recipients of a first cadaveric transplant with a PRA of more than 50% have a 6% lower graft survival at one year as compared to recipients with a PRA of less than 10% (Cecka 1996).

Non-immunological factors

It is likely that non-immunological factors also contribute significantly to the progression of CAN. An analysis of pooled UNOS data has shown that the survival of kidneys from living-unrelated and one-haplotype match living-related donors (i.e. one half of the HLA genes of the donor and recipient are identical) is extremely good and much superior to the survival of kidneys from a cadaveric source with similar degrees of HLA match. This suggests that the condition of the kidney at the time of transplantation is an important, alloantigen-independent predictor of CAN (Terasaki *et al* 1995). Organs from cadaveric donors in their late 50s and 60s may have age-related impairment of function, particularly when the cause of death is vascular rather than traumatic. Older kidneys may also be more susceptible to acute tubular necrosis. CAN is more likely to develop in kidneys from donors who are female, black and either older than 60 years old or less than 3 years old (Chertow *et al* 1996). 'Nephron dose' may be the explanation for this observation, as discussed later in this section. It is relevant to note that the mean age of cadaveric kidney donors in the UK has increased steadily from 37 years to 43 years over the past decade (UK Transplant 2001).

Retransplantation experiments in animals have demonstrated the importance of alloantigen-dependent events in the development of chronic graft deterioration but they suggest also that a 'point of no return' exists, before which acute rejection or early chronic rejection can be reversed and beyond which the process of fibrosis becomes independent of the stimulus and progresses inexorably (Yang *et al* 1995; Tullius *et al* 1997; Tullius *et al* 1998). Yang *et al* studied the *in situ* expression of platelet-derived growth factor (PDGF-beta), a cytokine that is produced in response

to wound injury. Rat allografts (Fischer 344 to Lewis) were retransplanted into syngeneic Fischer recipients at 12 weeks (once CAN had developed) and evaluated for glomerular PDGF-beta mRNA expression after a further 12 weeks. Expression was significantly higher in allografts (Fischer to Lewis) as compared to isografts (Lewis to Lewis), but the difference was less marked in retransplants (Fischer to Lewis and then back to Fischer), providing evidence of the importance of alloantigen-dependent factors in the progression of CAN. Tullius *et al* retransplanted acutely rejecting rat kidney grafts (Lewis to BNF1) into syngeneic (Lewis) recipients. For the allografts with massive cellular infiltrates and necrosis, rejection could not be reversed by retransplantation and the syngeneic recipients later died of renal failure. In contrast, for allografts that were less severely damaged by rejection a complete reversal of the rejection process occurred following retransplantation and this was accompanied by a significant fall in urinary protein excretion.

The inexorable decline in function following retransplantation of the more severely damaged rat allografts is not fully explained. One theory relates to adaptive changes in renal haemodynamics (Brenner *et al* 1992). A critical reduction in the number of functioning nephrons may produce hypertrophy of residual nephrons secondary to increased intravascular pressure and flow. This process is termed *glomerular hyperfiltration* (Gottschalk 1971) and it would of course be exacerbated in the presence of *systemic hypertension*. The permselectivity of the glomerular basement membrane (GBM) is reduced, so leading to an increase in urinary protein excretion. Accumulation of protein deposits within the mesangium stimulates mesangial cell proliferation and deposition of matrix with subsequent glomerulosclerosis (Couser *et al* 1975). The associated loss of glomerular function exerts a positive feedback

stimulus that produces compensatory hyperfiltration in less affected glomeruli, contributing in turn to further glomerular injury and destruction. Hyperfiltration injury has been reported in a number of experimental and clinical models in which there is nephron loss (Brenner *et al* 1982). One way of estimating its importance in the transplant setting is to examine the effect of donor-recipient size mismatching on graft survival. Published studies have given conflicting results (Miles *et al* 1996; Lee *et al* 1997; Kim *et al* 2001). The glomerular pathology that is associated with hyperfiltration, namely focal glomerular sclerosis, is not prominent in chronically rejecting renal allografts, suggesting that hyperfiltration does not in fact play a major role in the development of CAN. CsA promotes vasoconstriction of the afferent glomerular arteriole and may therefore protect functioning nephrons from hyperfiltration injury at the expense of glomerular ischaemia. Focal glomerular sclerosis was not a frequently reported histopathological finding in the pre-CsA era, perhaps contradicting this hypothesis.

Hyperlipidaemia may contribute to the development of CAN. Foam cells and apolipoprotein deposits are seen commonly in the vascular lesions of chronic rejection. Prospective and retrospective studies have documented an association between CAN and raised serum levels of cholesterol and/or triglycerides (Guijarro *et al* 1995; Fernandez-Miranda *et al* 1997; Roodnat *et al* 2000). Randomised trials in human cardiac allograft recipients have shown that 3-hydroxy-3-methylglutaryl-coenzymeA (HMGCoA) reductase inhibitors may be effective in limiting cardiac allograft vasculopathy (Kobashigawa *et al* 1995). These are discussed in more detail in Section 4.6.6.

The expression of MHC class II antigens in renal allografts has been shown to increase during *CMV infection* (Lemstrom *et al* 1994; von Willebrand *et al* 1986). The relationship between CMV infection and long-term graft survival may be causal or simply an epiphenomenon resulting from the dual association of CMV infection with acute rejection (which typically precedes infection) and increased donor age, both of which are considered to be independent predictors of poor long-term graft survival (Cecka 1997).

Brain death and organ reperfusion (the flow of recipient blood through a transplanted organ that has been preserved at low temperature following its removal from the donor) are associated with an increase in both MHC class II expression and cytokine production. Importantly, these stimuli are of course avoided in living donor transplantation. The duration of cold injury to cadaveric grafts in the UK approaches 24 hours on average. Immunological upregulation may be caused by the dramatic catecholamine release that accompanies brain death and the ischaemic and oxidative injury that is associated with organ reperfusion (Pratschke *et al* 1999).

4.2 Histopathology of CAN

The histopathological features of CAN are generally non-specific. *Vascular* changes include concentric intimal thickening of arteries and arterioles with infiltration of macrophages and lymphocytes and migration of myofibroblasts from the tunica media. Multilayering of peri-tubular capillaries may also be seen. *Glomerulopathy* comprises wrinkling and collapse of the glomerular tuft, mesangial matrix expansion and hypertrophy of remnant glomeruli. *Tubular atrophy* and *interstitial fibrosis* are prominent features, the latter resulting from deposition of extracellular matrix proteins such as collagen type IV and laminin. Immunofluorescence studies commonly reveal linear fibrin and granular IgM, C1q and C3 deposits along capillary walls.

The Banff classification represents an organised and consistent approach to the classification and grading of the various structural lesions that occur in renal allografts, as agreed by an international committee of specialists (Racusen *et al* 1999). The severity of the histopathological features is considered within the Banff international classification system for CAN (category V, grades 1-3), added weighting being given to tubulo-interstitial changes as these are of greater prognostic significance and interpretation is less prone to sampling error. The histopathological features of chronic rejection in other solid organ transplants are similar to those of CAN, in that there is obliterative fibrosis of hollow structures within the graft, be they blood vessels, bronchioles, bile ducts or pancreatic ducts.

4.3 Pathophysiology of CAN

There are several theories concerning the pathophysiology of CAN. Essentially, renal injury of any kind promotes an immunological response with influx of inflammatory cells, release of chemical mediators (such as cytokines, enzymes and growth factors), proliferation of tissue fibroblasts and deposition of extracellular matrix proteins that results in scarring. The strength of this response is influenced by genetic factors (Section 4.4). A substantial loss of functioning renal mass promotes further renal damage through the effects of hypertension, proteinuria and dyslipidaemia (Mackenzie *et al* 1994; Benediktsson *et al* 1996). Transplants with a lower initial *nephron mass* (e.g. kidneys from paediatric donors, females, blacks and older donors with age-dependent glomerulosclerosis) are more susceptible to CAN (Chertow *et al* 1996).

The importance of *apoptosis* and premature *senescence* of allograft parenchymal cells in CAN has not been fully established (Wever *et al* 1998; Halloran *et al* 1999). Apoptosis describes an orderly sequence of events comprising recognition of cells that are expressing pro-apoptotic cell surface receptors, cessation of metabolic activity within these cells, disassembly of the cells and disposal of their contents (Steller 1995). The process applies ordinarily to cell populations with a high turnover rate (e.g. leucocytes and intestinal epithelial cells) but may also occur in cells that are the target of sub-threshold chemical or physical insults. Above the threshold, cellular disruption rather than apoptosis would be more likely to occur, with release of enzymes and inflammatory mediators that exacerbate cellular injury and lead ultimately to tissue *necrosis*. Thus apoptosis is *programmed* cell death, whereas necrosis describes unprogrammed destruction of contiguous cells in part of an organ

or tissue. Inducers of apoptosis include a family of lethal cytokines that activate a corresponding family of so-called 'death receptors'.

The relevance of *thrombophilia* (both congenital and acquired) to the development of CAN is not yet clear (Irish 1999). It is conceivable that the vascular endothelial damage seen in CAN is more likely to be complicated by thrombotic occlusion of arterioles in patients who have a coagulopathy, resulting in tissue ischaemia and more rapid disease progression. Fischereder *et al* tested 132 consecutive renal transplant recipients for protein C and S activity, lupus anticoagulant (LA) and the factor V Leiden (FVL) mutation. A total of 18 patients had a detectable abnormality (FVL mutation, 10; LA, 6; protein S deficiency, 2). The median graft survival for patients with thrombophilia was 30 months compared to 86 months for patients without thrombophilia ($p < 0.01$) (Fischereder *et al* 1998). A review of 109 renal transplant recipients performed by Ekberg *et al* found that the factor V Leiden mutation was an additional risk factor for early renal graft loss in patients with acute vascular rejection (Ekberg *et al* 2000). There is also evidence of an association between a prothrombin gene mutation and early graft thrombosis, although case numbers are small (Oh *et al* 1999).

Recent studies have examined plasma homocysteine (Hcys) levels in renal transplant recipients. Hyperhomocystinaemia is associated with premature vascular disease. Plasma levels depend to some extent on the activity of methyltetrahydrofolate reductase (MTHFR), an enzyme that catalyses the remethylation of Hcys to methionine. Mutations of the MTHFR gene may result in the synthesis of a more thermolabile enzyme with reduced catalytic activity. It has been shown in renal transplant recipients that homozygosity for the C677T MTHFR gene mutation is

associated with higher homocysteine and lower folate levels in the serum (Fodinger *et al* 1999).

Other workers have demonstrated that CsA treatment is associated with increased platelet aggregation and impaired fibrinolysis (Vanrenterghem *et al* 1985; Malyszko *et al* 1996).

4.4 The role of cytokines and the importance of cytokine genotype in CAN

The cytokine family comprises a large and diverse group of low molecular weight, soluble proteins that regulate the immune response. They bind to cell surface receptors and can activate or suppress cells within which they are synthesised, neighbouring cells and distant cells (autocrine, paracrine and endocrine effects respectively) (Arai *et al* 1990). Typically, cytokines are produced for only brief periods, have high affinity for their cell surface receptors and display multiple synergistic and overlapping effects (pleiotropy). This may explain the poor efficacy of an immunosuppressive therapy that targets selectively the action of a single cytokine molecule, and yet by contrast a profound synergism when it is used in combination with other therapies (Ueda *et al* 1990).

Lymphokines are cytokines that contribute to allo-specific immunity. They are produced locally by activated helper T lymphocytes upon exposure to foreign MHC class II antigens expressed both within the allograft and by migrating donor dendritic cells in host lymphoid tissue. Lymphokines such as interleukin 2 (IL-2), IL-10 and transforming growth factor-beta (TGF-beta) promote differentiation and proliferation of lymphocytes and activate other cells including neutrophils and macrophages.

Cytokines that regulate natural immunity are produced by mononuclear phagocytes and are called monokines (e.g. interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF-alpha). Monokines serve as important co-stimulators of T cell activation during acute allograft rejection. They also mediate activation of endothelial cells (to facilitate leucocyte adhesion) and the coagulation cascade.

Interferon-gamma (IFN- γ) is a prime example of a third group of cytokines. These are produced by activated T cells and serve to stimulate macrophages and upregulate MHC class II antigen expression on endothelial cells.

Other important mediators of the immune response include adhesion molecules (selectins, integrins, vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM)), vasoactive hormones (endothelin, prostaglandins, platelet activating factor (PAF)) and growth factors (platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF)).

The *TGF-beta* family of cytokines regulates a number of processes including cell proliferation, angiogenesis, inflammation, immune regulation, and extracellular matrix production and remodelling (Kitamura *et al* 1997). TGF-beta does not act alone, rather it works in association with other factors such as PDGF and fibroblast growth factor (FGF) (Roberts *et al* 1990). TGF-beta exists in three isoforms in mammals: TGF-beta 1, 2 and 3. These perform similar functions *in vitro*, but TGF-beta 1 is the principal isoform involved in fibrogenesis *in vivo* (Border *et al* 1994). TGF-beta 1 is produced by a wide variety of cells including monocytes and lymphocytes, renal tubular cells and vascular endothelium. It is transported in the circulation within the alpha granules of platelets and is released in a latent form bound noncovalently to another dimer peptide, the latency-associated peptide (LAP). Active TGF-beta 1, a 25-kDa dimer protein composed of two subunits linked by a disulphide bond, is released by the action of enzymes such as thrombospondin. Decorin, a proteoglycan present in the extracellular matrix of most tissues, is thought to act as an effector molecule in a feedback loop regulating TGF-beta 1 production (Border *et al* 1994).

The chemoattractant and immunosuppressive properties of TGF-beta 1 are evident at low levels of expression. At higher levels of expression, TGF-beta 1 contributes to wound healing and fibrosis via fibroblast migration, collagen synthesis and inhibition of collagenases and other metalloproteinases. TGF-beta 1 autoinduces its own production, thus amplifying its biological actions. Sustained activity produces arteriosclerosis (through myofibroblastic transformation) and fibrosis, two of the key histopathological features of CAN (Border *et al* 1994).

There are several independent sources of evidence to support the notion that TGF-beta plays a central role in the development of allograft fibrosis.

Firstly, 'high producer' TGF-beta 1 genotypes in both the organ *donor* and the *recipient* are associated with chronic rejection in heart, lung and liver transplantation (Awad *et al* 1998; Bathgate *et al* 1998; Densem *et al* 2000). A study of 100 renal allograft recipients in Manchester revealed that all patients who developed CAN within 5 years of transplantation (n = 9) had a 'high producer' TGF-beta 1 genotype (Sankaran *et al* 1999). However, the frequency of the 'high producer' genotype in the remainder of the study population was not stated. 8 of the 9 donors were also 'high producers', supporting the notion that the magnitude of TGF-beta 1 production by cells of donor origin is also of prognostic importance.

Secondly, *in vivo* studies of TGF-beta gene transfection into porcine arteries have demonstrated that TGF-beta expression correlates with extracellular matrix synthesis and intimal hyperplasia (Nabel *et al* 1993). Transgenic mice that overexpress TGF-beta in hepatic tissue develop generalised tissue and organ fibrosis (Sanderson *et al* 1995). Similarly, the degree of glomerulosclerosis and tubulo-interstitial fibrosis in rodent models of glomerulonephritis is proportional to the amount of glomerular

TGF-beta activity (Yamamoto *et al* 1994). TGF-beta mRNA expression in the vascular wall of rat aortic allografts is increased markedly during the proliferation phase of chronic rejection (Little *et al* 1999).

TGF-beta 1 expression in the glomerular tuft and tubulointerstitium does not precede the development of CAN in all cases, but it is a consistent finding in more established lesions (Shihab *et al* 1995; Stegall *et al* 2001; Hueso *et al* 2001). Comprehensive gene expression profiles produced by GeneChip microarray technology have been invaluable in this area of research. Laboratory detection of fragments of DNA or RNA had depended until recently on the use of labelled DNA probes with complementary sequences. The probes are able to bind selectively to the fragments of specific interest. It is now possible to miniaturise this method, such that tens of thousands of specific DNA or RNA sequences can be detected simultaneously on a small glass or silica slide only 1-2 cm square (a microarray). The technology is relatively new, but reliability is improving with the advent of a photolithographic method of slide preparation (Aitman 2001).

Further evidence of the important role of TGF-beta 1 comes from *in vitro* studies of arteriosclerosis showing that intimal thickening of arterioles is less marked when production of TGF-beta 1 and platelet-derived growth factor (PDGF) is inhibited by rapamycin (Gregory *et al* 1993).

Strutz *et al* have demonstrated that TGF-beta 1 promotes transdifferentiation of tubular epithelial cells into mesenchymal cells (myofibroblasts) *in vitro*, which are capable of producing extracellular matrix proteins such as fibronectin and collagen type I (Strutz *et al* 2000).

Finally, neutralising anti-TGF-beta antibodies are able to suppress inflammation in an animal model of acute mesangial proliferative glomerulonephritis when administered at the time of disease induction. Extracellular matrix deposition and other histopathological features of the disease are markedly attenuated (Border *et al* 1990). The proteoglycan decorin, a matrix component that regulates TGF-beta activity, has been shown to inhibit extracellular matrix production in similar experiments (Border *et al* 1992).

TGF-beta 1 mRNA production is thought to be influenced by angiotensin II and oxidised low-density lipoproteins (Ketteler *et al* 1995; Ding *et al* 1997). Activated cytotoxic T lymphocytes that are exposed to TGF-beta 1 express a cell surface antigen (CD103) that binds to E-cadherin, an epithelial cell-specific adhesion molecule. TGF-beta 1 may therefore promote adhesion of T lymphocytes to tubular epithelial cells with subsequent tubulocytolysis (Hadley *et al* 1997).

Active TGF-beta 1 is often detectable in the plasma during episodes of acute rejection and CMV infection. It has been reported that 25% of renal allograft recipients have detectable plasma levels of TGF-beta 1 within the first post-transplant year (Williams *et al* 1995). TGF-beta 1 production is thought to be increased in patients treated with CsA (Ahuja *et al* 1995; Shin *et al* 1998) but not in those who receive tacrolimus (Hutchinson 1999). Other evidence suggests that the former observation may be an epiphenomenon resulting from a more enhanced release of platelet TGF-beta 1 in the presence of CsA (Brenchley *et al* 1998). The expression of receptors for TGF-beta 1 is increased in the presence of CsA (Hutchinson 1999) whereas tacrolimus disrupts TGF-beta 1 receptor activity by binding to one of its components (FK binding protein) (Okadome *et al* 1996).

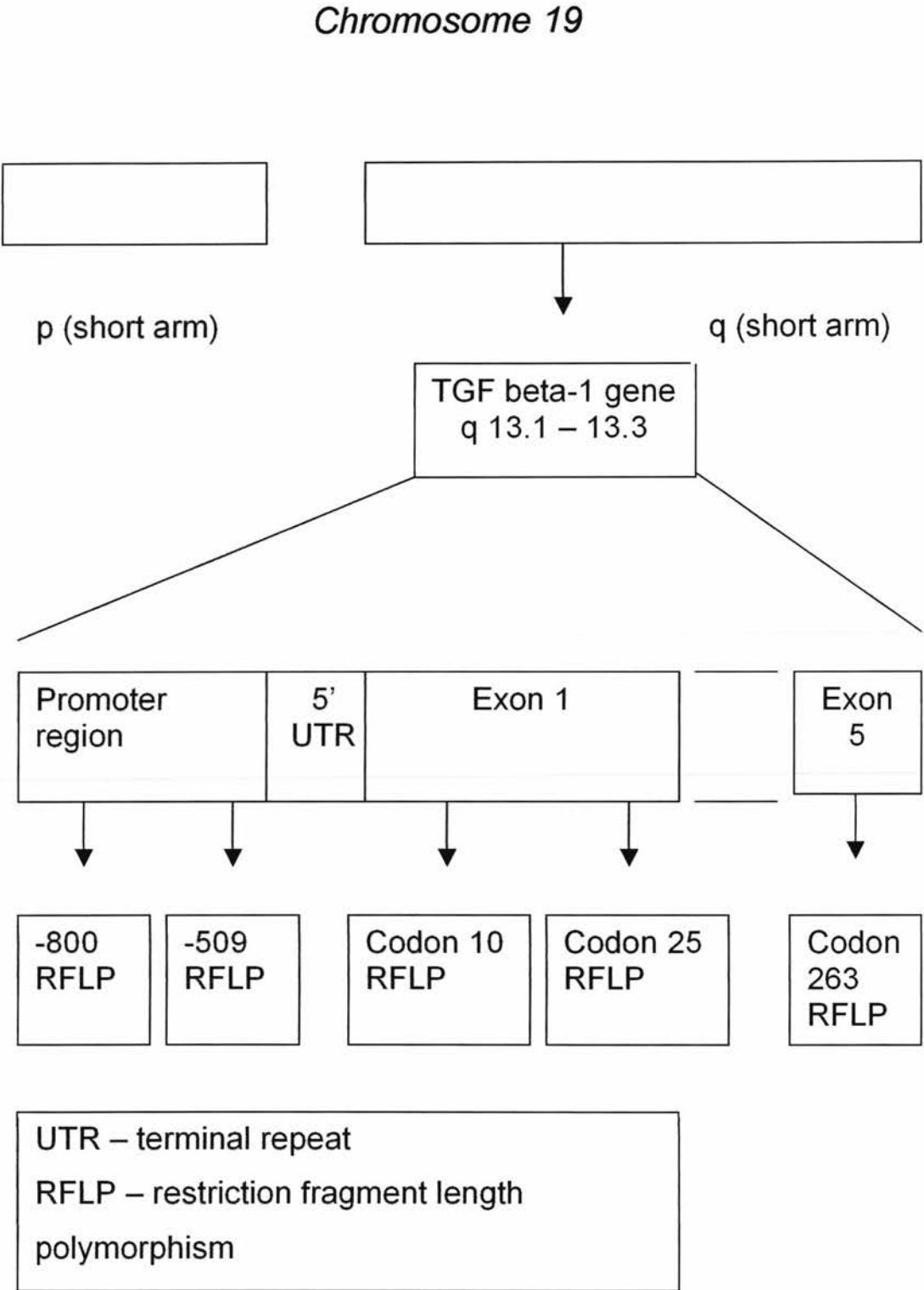
As discussed previously, the magnitude of cytokine release from activated lymphocytes (and hence the immunoresponsiveness of an individual) depends partly on genotype (Hutchinson *et al* 1998). A 'high producer' TNF- α genotype may be a predisposing factor for the development of acute allograft rejection, particularly in combination with a 'low producer' genotype for the anti-inflammatory cytokine interleukin-10 (Turner *et al* 1997). This is not a consistent finding, for example Marshall *et al* recently studied 209 cadaveric renal transplant recipients and were unable to demonstrate an association between recipient cytokine genotype and acute rejection within the first 30 days post-transplantation (Marshall *et al* 2000). This study can be criticised in terms of there being a high overall rate of acute rejection (perhaps 'masking' the truly 'high-risk' population) and a failure to analyse the effect on outcome of combinations of cytokine genotypes. The latter would have been difficult to perform given the limited size of the study population. A 'high producer' TGF-beta 1 recipient genotype may be associated with frequent and severe acute rejection episodes and chronic rejection (Sankaran *et al* 1999; Buscaroli *et al* 2001) but again there are other studies that have produced contradictory results. For example, a recent study by Lacha *et al* found that patients with a 'low producer' TGF-beta 1 genotype had the poorest clinical outcomes (number of acute rejection episodes and development of CAN), especially in association with a 'high producer' TGF-beta 1 *donor* genotype (Lacha *et al* 2001).

Polymorphisms in the promoter, enhancer and leader sequences of the TGF-beta 1 gene may influence TGF-beta 1 production (Figure 2). *Codon 10* encodes part of an alpha-helical structure that directs transport of the TGF-beta 1 protein through cells. In the allelic variant of codon 10, proline is exchanged for leucine resulting in

distortion of the alpha helix. The allelic variant of *codon 25* is associated with impaired cleavage of the leader sequence of TGF-beta 1, thereby preventing TGF-beta 1 release from cells.

A comprehensive knowledge of donor and recipient genotypes may serve in the future as a useful guide to the selection of an optimal immunosuppressive regimen for individual patients.

Figure 2. The location and structure of the TGF-beta 1 gene



4.5 Cyclosporin nephrotoxicity or CAN?

Cyclosporin (CsA), a cyclical polypeptide metabolite of the fungus *Trichoderma polysporum*, was the first of a generation of immunosuppressive agents that suppress selectively the activation of T cells. This effect is achieved primarily by impairing the autocrine production of T cell growth factors such as interleukin 2 (IL-2). Transcription of IL-2 is increased following dephosphorylation of nuclear factor of activated T cells (NFAT), which migrates to the cell nucleus and binds to the IL-2 promoter region. Dephosphorylation of NFAT is a rate-limiting step that requires calcineurin, a calcium-sensitive serine-threonine phosphatase. CsA binds to intracellular cyclophilins and the resultant complex binds to calcineurin, thereby inhibiting its activity. CsA has other immunomodulatory effects (some of which paradoxically enhance immunoactivation) that are less well defined.

It is extremely disappointing that the lower rates of acute transplant rejection seen after CsA was introduced to immunosuppressive regimens in the early 1980s have not led to a more substantial improvement in long-term renal allograft survival (Schweitzer *et al* 1991, Thorogood *et al* 1992). The prevailing view that CsA has an unfavourable effect on long term outcome has been called into question by recent data from the USA (Hariharan *et al* 2000). The issue is difficult to resolve for two main reasons.

Firstly, there is insufficient comparative follow-up data (beyond 5 years post-transplant) for groups of patients that have received different immunosuppressive regimens (CsA-based and others). A 10-year follow-up study reported by the European Multicentre Trial Group found no significant difference in graft attrition

rate after the first transplant year for prednisolone/ CsA and prednisolone/ azathioprine (AZA) regimens (Beveridge *et al* 1995). Similar findings have been reported by Vanrenterghem (Vanrenterghem *et al* 1996). A comparison of over 4,000 CsA-treated transplant recipients and over 10,000 historical 'controls' treated with AZA/ prednisolone (taking data from thirteen selected studies) found no significant difference in rates of graft failure or functional attrition (Lewis 1995). The latter finding is supported by a recent study by Velosa *et al* that reported a mean annual reduction in GFR of 3ml/min for both CsA and non-CsA treated groups of renal transplant recipients with CAN (Velosa *et al* 2001). It may be concluded, therefore, that CAN rather than chronic CsA nephrotoxicity is the pre-eminent pathological process preventing a more uniformly successful long-term graft outcome. However, there is some evidence that for individual patients the degree and variability of systemic exposure to CsA may be of prognostic importance. Several groups have reported an association between low cumulative CsA dose and CAN (Schroeder *et al* 1995; Citterio *et al* 1998; Opelz *et al* 2001). Retrospective data collected by the Collaborative Transplant Study Group indicate that graft survival is also reduced in patients with a very high CsA exposure, as measured by their weight-adjusted CsA dose at one year post-transplant (Opelz *et al* 2001). It has also been shown from pharmacokinetic studies that patients who display a high variability of CsA exposure have a significantly increased risk of developing CAN (Kahan 1998). Studies of elective post-transplant withdrawal of CsA have given conflicting results in terms of graft outcome (Kasiske *et al* 1993; Hollander *et al* 1995; Opelz 1995; Stoves *et al* 2002a). This is attributable in part to differences in case selection and CsA withdrawal protocols.

The second reason for uncertainty regarding the long-term effects of CsA relates to the difficulty in discriminating between the clinico-pathological features of chronic CsA nephrotoxicity and CAN (Mihatsch *et al* 1995). For example, both are associated with hypertension and the clinical features of CsA toxicity such as tremor, gingival hyperplasia and renal tubular hyperchloraemic acidosis may not be evident. Both processes involve blood vessels and the tubulointerstitial space and distinctive histopathological features (see below and Section 4.2) may be absent, such that only non-specific end-stage abnormalities such as arterial intimal fibrosis, arteriolar hyalinosis, intraglomerular fibrin thrombi, irregular tubular vacuolation and generalised interstitial fibrosis are seen. A consideration of some general points may be of diagnostic value. Firstly, CsA nephrotoxicity is predominantly an arteriolar lesion whereas CAN typically affects larger vessels. Secondly, CsA nephrotoxicity commonly involves the tunica media with myocyte loss and replacement by hyaline deposits, whereas CAN is primarily a proliferative intimal disorder with cellular infiltration. Glomerular features such as lobulation and reduplication of the capillary basement membrane are more typical of CAN. Finally, the presence of an interstitial infiltrate of inflammatory cells (lymphocytes, macrophages and plasma cells) would favour a diagnosis of CAN.

In attempting to discriminate between the two pathological processes, it must be recognised that CsA may itself contribute to the development and progression of CAN. Previous work in mice has shown that CsA inhibits the development of tolerance induction by mycophenolate mofetil (see Section 4.6.1) (Hao *et al* 1992). There is also some evidence that CsA has a stimulatory effect on B cells *in vitro*, thus promoting plasma cell differentiation and IL-6 release (Wortley *et al* 2001). The

effect of CsA on TGF-beta 1 production is discussed later in this section (and in Section 4.4).

What is the precise mechanism of 'pure' CsA nephrotoxicity? It is well known that treatment with CsA is associated with an *acute* dose-related decrease in renal function in both experimental animals and human volunteers (Bennett *et al* 1996). The rapid reversibility of this effect when CsA is withdrawn (Curtis *et al* 1986) suggests that it does not represent a primary toxic insult to renal tubular cells, rather an alteration in intrarenal vascular resistance that is mediated predominantly by vasoconstriction of the afferent glomerular arteriole. CsA is known to disturb many of the vasoactive systems that control renal vascular tone and GFR including nitric oxide, endothelin, prostaglandins and platelet activating factor (PAF), and it may also have a direct effect on the tonicity of vascular smooth muscle cells and mesangial cells (Cairns *et al* 1998). The histological features of acute CsA nephrotoxicity are consistent with acute ischaemic injury and include hyalinosis and myocyte necrosis of renal arterioles and isometric vacuolisation of renal tubular cells.

Chronic exposure to CsA may produce permanent nephrotoxicity. The histopathological features of chronic CsA toxicity in renal allografts include striped tubulointerstitial fibrosis, tubular atrophy and nodular hyperplasia of afferent arterioles (Feutren *et al* 1992). These features are seen first in the renal medulla with later extension to the medullary rays of the cortex. Klintmalm *et al* found more severe interstitial fibrosis and tubular atrophy in CsA-treated versus non-CsA-treated renal transplant recipients (Klintmalm *et al* 1984). The histopathological features correlated with CsA trough levels and the cumulative prescribed CsA dose during the

first 6 months post-transplant but were evident even in allografts of patients receiving doses of less than 4 mg/kg/day. CsA dose reduction or withdrawal is associated with a partial resolution of CsA-related arteriolopathy but persistence of the tubulointerstitial features (Bennett *et al* 1986).

Chronic CsA nephrotoxicity was described in *cardiac* allograft recipients as early as the mid-1980s (Myers *et al* 1984). Myers subsequently reported that the prevalence of ESRF within 10 years of heart transplantation was approximately 10% (Myers *et al* 1991). Glomerulosclerosis is a typical histopathological feature in addition to the vascular and tubulointerstitial changes that occur in renal allografts. It is likely that the kidneys of heart transplant recipients are chronically ischaemic, leading to upregulation of the renin-angiotensin system and consequently an exacerbation of CsA-induced renal fibrosis as discussed later in this section.

CsA nephrotoxicity is a major cause of progressive renal dysfunction in *lung* and *heart-lung* transplant recipients (Zaltzman *et al* 1992; Pattison *et al* 1995). In a study of 30 lung transplant recipients who were followed-up for a minimum period of 6 months, 50% developed hypertension, 30% had serum creatinine measurements of above 3 mg/dl (250 μ mol/l) and 2 reached ESRF.

Following *liver* transplantation, GFR decreases almost immediately to about 45-60% of normal but then remains stable for several years (Wheatley *et al* 1987; Platz *et al* 1994). A similar incidence and pattern of nephrotoxicity is described with the calcineurin inhibitor tacrolimus (FK506).

CsA is used in *bone marrow* transplantation to prevent graft versus host disease. In a histopathological study of CsA-treated bone marrow transplant recipients that included autopsy specimens, Dieterle *et al* found striped interstitial fibrosis and

arteriolopathy in 50% and 70% of cases respectively. The severity of these lesions correlated with the duration of CsA therapy. Serum creatinine measurements tended to rise by 40 - 80% within 3 months of commencing CsA and then stabilise (Dieterle *et al* 1990).

CsA has been used in the treatment of various *glomerular disorders*. Meyrier *et al* found that patients with minimal change nephrotic syndrome receiving CsA doses of up to 5.5 mg/kg/day did not develop histological features of CsA nephrotoxicity. Although similar results were reported for a group of CsA-treated patients with focal glomerulosclerosis (FSGS), renal function deteriorated in some patients (especially those with pre-existing renal dysfunction). This was due in part to exacerbation of the underlying renal pathology (Meyrier *et al* 1994).

Although relatively low doses of CsA are used in the treatment of *autoimmune disease* and rheumatoid arthritis, interstitial fibrosis and other changes of chronic nephrotoxicity tend to occur within 2 years of commencing treatment (Sund *et al* 1994). It has been shown in long-term studies that the histological features are progressive (Young *et al* 1994). The most frequently occurring lesions are glomerular collapse, arteriolar hyalinosis, tubular atrophy and interstitial fibrosis (Mihatsch *et al* 1994). A meta-analysis and morphological review of CsA-induced nephrotoxicity in conditions such as rheumatoid arthritis, psoriasis, atopic dermatitis and Crohn's disease found that the weighted percentage increase in serum creatinine was 17% in CsA-treated patients and 1.7% in 'control' patients who did not receive CsA (Vercauteren *et al* 1998). An incomplete reversal of renal dysfunction following withdrawal of CsA was reported in some patients. According to Feutren *et al*, a high

CsA dose (above 5mg/kg/day) and increasing patient age are the main predictors of chronic CsA nephrotoxicity (Feutren *et al* 1992).

The anti-fibrotic drug pirfenidone has been shown to decrease TGF-beta 1 expression and ameliorate fibrosis in the salt-depleted rat model of CsA nephrotoxicity (Shihab *et al* 2001). Salt depletion may upregulate the expression of angiotensin II-dependent lymphokines and fibroblast growth factors, thus providing a link between acute vasomotor changes and chronic tubulo-Interstitial damage (Wolf *et al* 1993). CsA increases the recruitment of renin-producing cells along the afferent glomerular arteriole (Tufro-McReddie *et al* 1993; Moore *et al* 1995). Johnson *et al* have demonstrated in rats that angiotensin II infusions can produce tubulo-Interstitial changes similar to those seen with chronic CsA nephropathy (Johnson *et al* 1992). Furthermore, the angiotensin II type I receptor is located in the outer medulla and the medullary rays, mirroring the distribution of lesions in the animal model of chronic CsA toxicity (Meister *et al* 1993).

Although renal vasoconstriction is seen as the most likely cause of the histopathological changes of CsA nephrotoxicity, *in vitro* studies have shown that other mechanisms may be important. For example, even small doses of CsA promote the synthesis of extracellular matrix components such as collagen type III (Ghiggeri *et al* 1994; Oleggini *et al* 2000). There is also evidence that CsA stimulates production of TGF-beta 1, a cytokine that has both immunosuppressive and fibrogenic properties (Shihab *et al* 1996) (see Section 4.4). TGF-beta 1 expression decreases in patients with CAN who undergo CsA withdrawal or CsA dose reduction (Wei *et al* 1998; Hueso *et al* 1998).

In addition to nephrotoxicity, CsA treatment is associated with hypertension and hyperlipidaemia. These are major risk factors for ischaemic cardiovascular disease, which has a major impact on patient survival and hence renal allograft survival. CsA-induced stimulation of the renal sympathetic nervous system may be one mechanism by which hypertension develops in recipients of non-renal organ transplants. It has been suggested that the denervation that occurs during procurement of a donor kidney ameliorates this effect in renal transplantats (Mark 1990) but the theory is refuted by strong experimental evidence and is therefore unlikely to be a significant factor (Remuzzi *et al* 1995).

CsA is metabolised extensively in the liver by the enzyme cytochrome P450-3A4. Metabolites are excreted primarily via the hepatobiliary system. The large inter- and intra-individual variability of CsA absorption (less marked for the microemulsion CsA formulation, *Neoral*®) makes dose monitoring difficult. Several metabolites of CsA (including M-17) may produce nephrotoxicity. Most of the laboratory assays that are used to measure the concentration of CsA in venous blood detect only the parent compound, and this is clearly an important limitation which needs to be recognised when using blood CsA concentrations as a guide to CsA dose adjustment.

4.6 Options for treatment of CAN

There are currently no effective strategies for the prevention or treatment of CAN in human subjects other than measures to minimise the impact of risk factors such as poor HLA matching, ischaemia-reperfusion injury, acute rejection, CMV infection, hypertension, hypercholesterolaemia and CsA nephrotoxicity (see Section 4.1). Clinical trials of immunosuppressive agents other than CsA will be discussed in this section, followed by a review of the role of ACE inhibitors and finally a summary of experimental approaches to the treatment of CAN.

4.6.1 Mycophenolate mofetil

Mycophenolate mofetil (MMF) is the morpholinylester of mycophenolic acid (MPA), a potent inhibitor of T and B lymphocyte proliferation. MPA also downregulates antibody production (of potential importance in the treatment of CAN) and the expression of cell-surface adhesion molecules that promote binding of lymphocytes to vascular endothelial cells at sites of inflammation (Grinyo 1999). It inhibits specifically the enzyme inosine monophosphate dehydrogenase, a critical, rate-limiting factor in the so-called '*de novo pathway*' of purine synthesis.

MMF is effective in preventing early, acute renal allograft rejection (Tricontinental Mycophenolate Mofetil Renal Transplant Study Group 1996 and 1998; European Mycophenolate Mofetil Cooperative Study Group 1995 and 1999). In the Tricontinental study, 503 newly transplanted patients were randomised to receive azathioprine (AZA) 100 – 150 mg/day, MMF 2g/day or MMF 3g/day in addition to prednisolone and CsA. During the first 6 months post-transplant, the incidence of biopsy-proven acute transplant rejection was reduced by approximately 50% in the

MMF 2g/day (19.7%) and MMF 3g/day (15.9%) groups as compared to the AZA group. There was also a significant reduction in treatment failure (persistent rejection, death, graft loss or early withdrawal with no prior acute rejection episodes). Graft function (intent-to-treat and on-study) was comparable in all three groups at 3 years, as was patient mortality.

In the European double-blinded, placebo-controlled study, 491 patients were randomised to placebo, MMF 2g/day or MMF 3g/day in addition to prednisolone and CsA. Significantly fewer patients had biopsy-proven rejection in the first 6 months post-transplant with MMF 2g/day (17%) and MMF 3g/day (13.8%) as compared to placebo (46.4%). An advantage in respect of other causes of early treatment failure (see above) was also reported. At 3 years post-transplant, MMF was associated with a 7.6% reduction in the incidence of graft loss (excluding graft loss resulting from patient death).

A meta-analysis of three major studies (European, Tricontinental and an American study) showed that graft loss due to rejection for a control group of patients receiving a regimen of steroids and CsA with or without AZA was 6.3% in the first post-transplantation year, whereas the groups receiving steroids, CsA and MMF at doses of 2g/day and 3g/day had corresponding graft loss rates of 2.6% and 3.5% respectively (Halloran *et al* 1997).

MMF has been used effectively in transplant recipients for whom there is a high risk of acute rejection and/or CsA nephrotoxicity (Agraz *et al* 1999; Esmeraldo *et al* 1999). It has also been used in combination with other agents for the treatment of refractory acute rejection (Mycophenolate Mofetil Renal Refractory Rejection Study 1996; Mycophenolate Mofetil Acute Renal Rejection Study Group 1998; Carl *et al*

1998). The main side effects of MMF are dose-related gastro-intestinal symptoms including nausea, abdominal pain and diarrhoea and myelosuppression.

MMF has reversed some of the histopathological features of CAN in both renal and aortic allograft models of chronic rejection in the rat (Raisanen-Sokolowski *et al* 1994; Azuma *et al* 1995). A reduction in interleukin-2 expression was documented in the former study. Therapeutic concentrations of MMF inhibit the growth of fibroblasts in cell culture studies. This effect is mediated by an increase in apoptotic activity (Zeier *et al* 2002). MMF may also prevent the progression of renal insufficiency in rats that undergo 5/6 renal ablation (Romero *et al* 1999). There are obvious limitations associated with animal model experiments and long-term outcome data are generally unavailable.

Recent pilot studies have addressed the clinical effectiveness of MMF in the treatment of established CAN. The choice of MMF may have been encouraged by evidence from earlier studies that late introduction of AZA (another antiproliferative agent) to dual therapy (CsA/prednisolone) regimens had a beneficial effect on graft outcome (Rocher *et al* 1989; Kliem *et al* 1998). Rocher *et al* studied 21 patients with CAN (not biopsy-proven in all cases) in whom AZA was commenced at 17.8 +/- 2.8 months post-transplantation. CsA dose was not reduced. There was an improvement in renal function following the introduction of AZA ($p < 0.01$) with no significant change in blood CsA trough levels. Kliem *et al* reported a favourable response to AZA in combination with CsA dose reduction. The latter is of course a potential confounding factor. Both studies are weakened by the absence of a control group and Kliem's study was in part retrospective.

Weir *et al* studied the clinical course of 28 CsA-treated renal allograft recipients with suspected CAN (diagnostic confirmation from transplant biopsy was not obtained in all cases) whose immunosuppressive regimen was changed at 24.3 ± 7.7 months post-transplantation (Weir *et al* 1997). MMF 2g/day was substituted for AZA and the CsA dose was halved. The dose of MMF was reduced subsequently in 8 patients because of gastrointestinal toxicity and/or anaemia. No patients received angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers. Renal function improved in 21 patients according to measurements of serum creatinine and there was a significant reduction in the mean serum cholesterol concentration. Not all patients had biopsy-proven CAN and it is not clear in what proportion of patients there was histological evidence of co-existent acute rejection. There were no control patients and the majority of study participants were male African-Americans. MMF conversion was initiated at different times post-transplantation across the study population. Follow-up was of variable duration, though generally of sufficient length to allow adequate assessment of outcome. A reliable method of measuring GFR was not employed.

Islam *et al* studied 43 transplant recipients with CAN (not all biopsy-proven) who were treated with MMF and reduced dose CsA to achieve a trough blood CsA level of 50 ng/ml (Islam *et al* 1998). The mean observation period was 15 (6-22) months. MMF was discontinued because of diarrhoea in 3 patients and infection and anaemia in 2 patients. Two patients suffered graft loss as a result of worsening chronic rejection. There was a significant improvement in mean serum creatinine from 3.14 ± 1.0 to 2.57 ± 0.82 mg/dl ($p < 0.01$). Once again, the study was small and uncontrolled. Several patients were excluded from the final analysis because of insufficient follow-

up. Not all study participants had biopsy-proven CAN. Renal function was measured using serum creatinine rather than isotope methods. There was no reference to the use of statin or ACE inhibitor medications during the study period.

Two earlier American studies in which CsA was not simultaneously reduced failed to demonstrate a significant benefit from the addition of MMF, suggesting that CsA dose reduction may have been the key component of Weir's regimen (Glicklich *et al* 1998; Smith *et al* 1997). The latter could be explained readily in terms of a favourable alteration in renal haemodynamics as CsA is known to produce vasoconstriction of afferent glomerular arterioles (Curtis *et al* 1986). Evidence in support of this theory is provided by a study of patients with biopsy-proven CsA nephrotoxicity without CAN by Houde *et al*. This demonstrated that conversion from a CsA/ prednisolone regimen to a MMF/ prednisolone regimen at 57 (12-109) months post-transplant was safe and led to improved graft function and better control of hypertension and hyperlipidaemia (Houde *et al* 2000). The same approach has been successful in CsA-treated patients with progressive chronic renal allograft dysfunction (Suwelack *et al* 2001; Dudley 2002). The Creeping Creatinine Study has examined the effect of introducing MMF followed by complete withdrawal of CsA in patients with biopsy-proven 'chronic allograft dysfunction' (this is clearly a misnomer and we await clarification in a forthcoming full publication). It is unique in having a control arm. Preliminary results show that renal function stabilised in 58% of patients receiving MMF in place of CsA (n = 73) as compared to only 28% of controls (n = 70) (Dudley *et al* 2002).

The results of other recent studies indicate that MMF may have a *direct* effect on the progression of chronic renal allograft dysfunction in patients with CAN. A

retrospective registry-based study of 66,774 renal transplant recipients (1988-1997) by Ojo *et al* found that MMF reduced the risk of CAN by 27% compared to AZA (Ojo *et al* 2000). This was not due entirely to a concomitant reduction in the number of acute rejection episodes. Death-censored graft survival for patients receiving MMF was significantly higher than for those receiving AZA (85.6% vs 81.9% after 4 years). As with all retrospective analyses, there are many potential sources of bias in this study that may not have been considered. Fritsche *et al* studied 44 patients with a diagnosis of chronic rejection in whom MMF treatment was initiated at 5.8 ± 4.8 years following renal transplantation (Fritsche *et al* 1998). MMF was substituted for AZA in the majority of cases. The overall rate of decline in GFR was reduced significantly during the follow-up period (10.1 ± 4.9 months). A reduction in MMF dose was necessary in 10 patients, mainly because of gastro-intestinal side effects. The study was partly retrospective and there were no control patients. Not all patients had previously been taking CsA. MMF conversion was initiated over a wide-ranging period post-transplantation and follow-up was of variable duration. It was not made clear how GFR had been calculated and there was no reference to concomitant use of statin or ACE inhibitor medication, both of which may have influenced clinical outcome. In an uncontrolled prospective study performed by Kliem *et al*, MMF 2g/day was added to a baseline regimen of prednisolone and CsA in 40 renal transplant recipients with CAN at a median of 82 (11-192) months following renal transplantation (Kliem *et al* 1999). Follow-up was of extremely variable duration (0.25 – 14 months). There were no episodes of acute rejection but 3 patients lost their graft as a result of persisting CAN. Of the 26 patients who were followed for more than 5 months, 15 showed stable or improved graft function (although this was not

measured by a reference method). Once again, there was no information about the use of statin or ACE inhibitor medications during the study period. MMF was discontinued in 6 patients (permanently in 3 cases) and the dose reduced in a further 8 patients because of gastro-intestinal side effects, leucopaenia and anaemia. In another uncontrolled study of 31 allograft recipients with biopsy-proven CAN who commenced MMF treatment at 52 ± 38 months post-transplant, 23 patients showed stability or improvement of renal function according to serum creatinine measurements (Campistol *et al* 1999). This change was not accompanied by a reduction in arterial hypertension or proteinuria. The mean trough CsA blood concentration for the group as a whole was lower at the end of the study period than at the beginning, but a sub-group analysis of patients in whom CsA trough levels had remained stable gave similarly encouraging results. Finally, a prospective, multi-centre study in Spain involving 122 CsA-treated patients with biopsy-proven CAN showed that the addition of MMF (replacing AZA in a half of cases) protected against graft loss, although once again there was no control group (Gonzalez Molina *et al* 2001). The majority of patients had Banff grade II or grade III transplant biopsy scores. Renal function was measured by serum creatinine. Multiple regression was used to calculate the slope of the 'reciprocal of creatinine versus time' (ROCT) plots for the pre-intervention and post-intervention periods. The median duration of follow-up was 18 months. MMF was discontinued in 18 patients because of unwanted side effects. The median values of the ROCT slopes for the whole group before and after the introduction of MMF were -2×10^{-4} and -7×10^{-6} (units not provided) respectively ($p < 0.001$). The difference remained significant when only

AZA-treated patients were considered and also when the analysis was limited to the 65 patients who had no change in CsA trough levels throughout the study period.

In summary, the majority of reported studies of MMF +/- CsA dose reduction for the treatment of CAN, though encouraging, are weakened by small size, loose inclusion criteria, use of suboptimal methods of measuring kidney function and a failure to include 'control' patients who do not undergo immunosuppression conversion. The latter is essential if bias relating to the potential benefits of study participation is to be minimised. These issues are addressed further in Chapter 4.

4.6.2 Tacrolimus (FK506)

Tacrolimus (FK506) is a macrolide metabolite of the fungus *Streptomyces tsukubaensis*. It inhibits T cell activation and T cell-dependent proliferation of B cells, principally through its ability to bind to intracellular immunophilins such as FK binding protein 12 (FKBP12). The resultant complexes bind specifically and competitively with calcineurin, thereby blocking activation of the NFAT pathway. Tacrolimus suppresses T cell activation in other ways such as blockade of cytokine receptor expression on target cells (Mori *et al* 1997). Oral absorption of tacrolimus is incomplete and there is a high degree of pre-systemic metabolism, principally by the cytochrome P450 enzyme CYP3A4 contained within enterocytes. The main metabolic pathways are hydroxylation and demethylation. The immunosuppressive activity of the parent compound far exceeds that of its metabolites.

Several studies have demonstrated that tacrolimus is a safe and effective immunosuppressant for renal transplant recipients (Jensik *et al* 1998; Mayer 1999). The incidence of both acute and chronic rejection is less than for CsA-based

regimens, although the trend for chronic rejection has not reached statistical significance even in large studies with several years of follow-up (Mayer *et al* 1997; Mayer 1999; Pirsch 2000). Patient and graft survival rates are comparable to those reported with CsA. However, if crossover due to rejection is considered as graft loss, 3-year graft survival is significantly higher in patients treated with tacrolimus (81.5% vs 70%, $p<0.05$) (Mayer 1999).

Tremor, diarrhoea and glucose intolerance are some of the more common adverse effects of tacrolimus. There is a lower incidence of acne, hirsutism and gingival hyperplasia in comparison to CsA-treated patients (Mayer *et al* 1997). Successful withdrawal of steroid treatment has been possible in a large proportion of patients treated with tacrolimus (Cronin *et al* 1997; Shapiro *et al* 1998; Buell *et al* 2000).

The most comprehensive outcome data for patients treated with CsA-based and tacrolimus-based immunosuppression regimens are provided by the US Kidney Transplant Registry. A review of over 38,000 CsA-treated and 500 tacrolimus-treated patients indicated a significant graft survival advantage for the latter group (Gjertson *et al* 1995). One-year graft survival in the CsA and tacrolimus groups was $86.6 \pm 0.2\%$ and $91.1 \pm 1.3\%$ respectively. Graft half-life estimations were approximately 9 years with CsA and 14 years with tacrolimus ($p<0.05$). The finding of superior graft survival with tacrolimus-based immunosuppression is supported by data from a prospective randomised study reported by Laskow, who also demonstrated that graft *function* at 3 years post-transplant was superior in tacrolimus-treated patients (Laskow 1999).

It has recently been reported by the European Tacrolimus Renal Registry Study Group that conversion from CsA to tacrolimus at the time of a first acute rejection

episode facilitates reversal of rejection and subsequently provides effective secondary prevention (Briggs 2000).

Pilot studies of substituting tacrolimus for CsA in patients with CAN have been encouraging, although the findings are inconclusive because of their small size, the absence of control groups and suboptimal measurement of transplant function. Of 14 renal allograft recipients who were converted to tacrolimus at a median of 1,750 days post-transplant with a mean pre-conversion GFR of 21.4 ± 9.1 ml/min, 5 showed stability or improvement of function (Morris-Stiff *et al* 1998b). Other studies have produced similar results but sample sizes have again been relatively small (Manu *et al* 1999).

4.6.3 Rapamycin

Rapamycin (RPM) reduces proliferation of T lymphocytes, B lymphocytes and some non-lymphoid cells by reducing cyclin-dependent kinase activity (Halloran 1996). For example, *in vitro* studies have shown that RPM inhibits endothelial cell and smooth muscle cell proliferation (Gregory *et al* 1993; Morris *et al* 1995). RPM binds to the mammalian target of rapamycin (mTOR) and blocks both the costimulation cascade that augments interleukin-2 gene transcription (in the G0 to G1 transition phase of the cell cycle) and the cytokine transduction pathway (in the late G1 phase) (Abraham 1998).

There is as yet no reported clinical experience of RPM in the treatment of patients with CAN. In a rat aortic graft model of chronic rejection, RPM inhibits the development of chronic vascular changes (endothelial denudation, subendothelial infiltration with inflammatory cells and smooth muscle proliferation) in contrast to

the more modest effects of CsA (Belitsky *et al* 1993). Schmid *et al* examined the effects of RPM on transplant vasculopathy in long surviving F344 rat heart allografts transplanted heterotopically into Lewis rat recipients (Schmid *et al* 1995). RPM was administered intraperitoneally for the first 14 days post-transplant in groups 1 and 2 (0.5 and 2 mg/kg/day) and daily throughout the follow-up period in groups 3 (0.5 mg/kg/day) and 4 (5 mg/kg/day for 14 days, followed by a maintenance dose of 2.5 mg/kg/day). Grafts were removed from animals in group 1 at 50, 75, 100, 150 or 200 days post-transplant. Animals in the other groups underwent nephrectomy at 100 days post-transplant. Infiltrating cell populations and patterns of cell surface molecule expression were characterised and compared between groups. In group 1, the incidence of transplant vasculopathy increased in proportion to the age of the graft. An infiltrate of cells expressing MHC class II antigen and upregulated expression of adhesion molecules and cytokines were other prominent features. Animals receiving a higher maintenance dose of RPM had a lesser degree of transplant vasculopathy and inflammatory cell infiltrate.

4.6.4 Corticosteroids, azathioprine and antilymphocyte antibody prophylaxis

There is no consensus regarding the effect on late renal allograft loss of corticosteroid therapy. A study by Hollander *et al* of prednisolone withdrawal at one year post-transplant reported an increased incidence of acute rejection episodes and post-withdrawal proteinuria (Hollander *et al* 1997). This approach may be more successful in combination with more potent immunosuppressive regimens.

Several studies have not shown a significant reduction in the incidence of CAN with addition of AZA to a dual therapy (CsA/ prednisolone) regimen, although there is a

trend towards improved graft survival (Kunz *et al* 1997; Amenabar *et al* 1998). More encouraging study data for patients with established CAN were presented in Section 4.6.1.

A meta-analysis of trials of antilymphocyte antibody prophylaxis has shown that the graft survival rate at 2 years post-transplant is 5% higher than for an untreated control group (Szczzech *et al* 1997), perhaps implying a lower incidence of CAN. A 15-year prospective randomised controlled single-centre trial involving 123 consecutive adult cadaveric renal transplant recipients produced similar short-term results, but there was no significant long-term (beyond 5 years) graft survival advantage for patients receiving prophylactic antibody therapy (Cantarovich *et al* 2002). A recent meta-analysis of interleukin-2 receptor monoclonal antibody (basiliximab or daclizumab) prophylaxis showed a 22% reduction in graft loss at 1 year but this was not statistically significant (Cockwell *et al* 2002).

4.6.5 Angiotensin converting enzyme inhibitors and angiotensin II receptor antagonists

Angiotensin II (Ang II) is thought to play an important role in producing the vascular lesions that are associated with CAN in addition to its role in the pathogenesis of chronic CsA nephrotoxicity (as discussed in Section 3.5). There is experimental evidence that Ang II promotes myofibroblastic transformation of endothelial cells, probably via activation of TGF-beta 1 (Kagami *et al* 1994) and endothelin (ET-1) (Millgard *et al* 1998).

Angiotensin-converting enzyme (ACE) inhibitor preparations lower both systemic and intraglomerular pressure (Bochicchio *et al* 1990) and may also reduce oxidative stress (Calo *et al* 2001). There is evidence that their use in patients with CAN is

associated with a reduction in proteinuria and a slower rate of decline in renal allograft function (Barnas *et al* 1996; Lin *et al* 2001). A retrospective study by Barnas *et al* found significantly lower urinary protein excretion (fractional excretion 69 ± 13 mg/l at 12 months compared to 57 ± 16 mg/l at the start of treatment) and a less rapid decline in allograft function (-9 ± 1.2 ml/min/year for the 12 months *before* starting treatment compared to -4.8 ± 1.3 ml/min/year for the 12 months *after* starting treatment) with ACE inhibitor therapy. The study had a number of methodological flaws, for example a transplant biopsy was not performed in some cases and patients were used as their own controls.

Angiotensin II receptor antagonists (AT1-RAs) produce a similar response to ACE inhibitors both experimentally (Furukawa *et al* 1996) and in clinical practice (Omoto *et al* 2001). Furukawa *et al* used the AT1-RA TCV-116 in a murine model of cardiac transplantation. Hearts of DBA/2 mice were transplanted heterotopically into B10.D2 mice. Recipients were treated with oral TCV-116, captopril or vehicle only. At 70 days post-transplantation there was a significant reduction in the severity of graft coronary arteriopathy in the TCV-116 group and captopril groups compared to the vehicle group ($p < 0.001$ and $p < 0.005$ respectively). Fibrotic lesions of the left ventricle were reduced significantly in the TCV-116 and captopril groups as compared to mice receiving vehicle only. Omoto *et al* studied 62 proteinuric renal transplant recipients, 28 of whom had CAN (a subgroup analysis of these patients was not performed). The AT1-RA candesartan cilexetil was commenced at a dose of between 4 and 12 mg daily. There was a significant reduction in proteinuria within 2 months of starting treatment that was sustained during follow-up, but serum creatinine measurements continued to show a rising trend. The response may have

been linked to an overall reduction in systemic blood pressure rather than a specific intrarenal effect of candesartan.

4.6.6 Statins

Statin therapy was discussed briefly in Section 4.1. There is evidence from experimental studies in cardiac and renal allograft recipients to support its role in the treatment and prevention of chronic rejection.

Kwak *et al* demonstrated recently that statins (HMG-CoA reductase inhibitors) have immunomodulatory effects (Kwak *et al* 2000). They suppress MHC class II antigen-mediated T cell activation directly through inhibition of the inducible promoter IV of the transactivator CIITA. This effect is observed in cell culture studies of primary human endothelial cells, monocytes and macrophages. However, randomised clinical studies have failed to demonstrate an effect of statins on acute renal allograft rejection (Holdaas *et al* 2001, Kasiske *et al* 2001).

Ji *et al* recently studied the efficacy of pravastatin for the prevention of CAN in rat kidney allografts (F344 to Lewis) (Ji *et al* 2001). Recipients were divided into 3 groups: syngeneic (Group 1); allogeneic - CsA treatment alone (Group 2); and allogeneic - CsA plus pravastatin treatment (Group 3). Renal function was assessed by serum creatinine at 130 days post-transplantation. Expression of anti-apoptotic Bag-1 products was analysed by Western blot. Group 1 rats had no histological evidence of CAN. Group 2 and 3 rats showed vascular obliteration, tubular atrophy, glomerular sclerosis and interstitial fibrosis, however these features were significantly less prominent in the pravastatin-treated group. There was also a lower mean serum creatinine and a greater mean upregulation of Bag-1 expression in

comparison to Group 2. The latter observation suggests that pravastatin may have a beneficial anti-apoptotic effect.

Jaeger *et al* compared a combined regimen of a statin and weekly extracorporeal H.E.L.P (Heparin-mediated Extracorporeal LDL/fibrinogen Precipitation) apheresis to statin therapy alone in two matched groups of human cardiac allograft recipients (Jaeger *et al* 1997). Graft vessel disease (GVD) was assessed by coronary angiography over a mean follow-up period of 3.6 years. GVD was apparent in only one patient who received the combined treatment, compared with a total of seven patients in the monotherapy group. The combined treatment was associated with a marked reduction in plasma levels of low density lipoprotein (LDL) cholesterol, lipoprotein A and fibrinogen. There were no reported adverse effects of apheresis, in particular there was no increase in the incidence of sepsis.

In a study by Kobashigawa *et al*, consecutive cardiac allograft recipients were assigned randomly to a pravastatin treatment group or a control (no pravastatin treatment) group (Kobashigawa *et al* 1995). Twelve months following transplantation, the pravastatin treatment group had a lower mean serum cholesterol, a lower incidence of allograft coronary vasculopathy (as determined by angiography and histopathological examination of autopsy specimens) and superior allograft survival. In addition, intracoronary ultrasound studies performed at one year post-transplantation revealed lesser degrees of intimal thickening in pravastatin-treated patients.

It remains to be seen if statin therapy will be effective in prolonging graft survival (by reducing the incidence of CAN and/or cardiovascular mortality) in human renal allograft recipients.

4.6.7 Dietary protein restriction

Modest dietary protein restriction improves glomerular permselectivity and may reduce the rate of deterioration in graft function, possibly via suppression of the renin-angiotensin system (Rosenberg *et al* 1995). There has not been a large randomised controlled trial of dietary intervention in patients with established biopsy-proven CAN. However, a randomised study of patients with advanced chronic native renal dysfunction (Modification of Diet in Renal Disease Study) provided some evidence that dietary protein restriction in the order of 0.6 g/kg/day can slow the rate of decline in GFR (Levey *et al* 1996). This effect was shown to depend on achieved rather than prescribed levels of protein intake. Diabetic patients were not included in the analysis. Concerns about suboptimal nutrition were not addressed in the study and this is probably the main reason for the reluctance of many physicians to follow this approach in their clinical practice. A small, uncontrolled retrospective study of dietary protein restriction in patients with chronic rejection showed a slowing of disease progression according to pre- and post-intervention ROCT plots (Salahudeen *et al* 1992).

4.6.8 Experimental therapies

The following approaches to the prevention and treatment of CAN have been pursued in the field of animal research. Many of the experimental agents have not been used in clinical practice because of concerns about safety, efficacy, practicability and cost-effectiveness.

Immunotolerance

Immunotolerance is the 'Holy Grail' of therapeutics in allogeneic transplantation. It would by definition eliminate the need for maintenance anti-rejection medication. Researchers have looked at many different components of the immune response and used a number of experimental strategies including T cell depletion, T cell costimulatory pathway blockade, establishment of microchimerism (the presence in the recipient of a small number of donor lymphoid cells that circulate without being challenged by the host immune system) and the development of 'knockout gene' (e.g. cytokine and complement genes) animal models. A global Immune Tolerance Network (www.immunetolerance.org) has been created to facilitate and coordinate the development of effective strategies for achieving immunotolerance in man. The following is a summary of the experimental evidence that *bone marrow transfusion* and *costimulatory pathway blockade* may be effective in preventing CAN.

The first *blood transfusion* effect to be described was an experiment of nature. Freemartin cattle dizygotic twins share a common placenta. It was demonstrated that such twins had haemopoietic chimerism (Owen 1945). A skin graft from one twin was not rejected by the other, thus contradicting the view that immunological reactivity to foreign tissue and proteins was rigidly predetermined (Anderson *et al* 1951). The discovery in mice of tolerance to skin grafts following neonatal exposure of the host to donor leucocytes can be regarded as the first blood transfusion experiment (Billingham *et al* 1953). The effect of transfusing blood from a 'third party' source prior to kidney transplantation in humans remains uncertain (Opelz *et al* 1973). Salvatierra *et al* studied the effect of *donor-specific* transfusion (DST) in living-related kidney transplants (Salvatierra *et al* 1987). Their key finding was that

early graft loss as a result of acute rejection was virtually eliminated in one HLA haplotype mismatch donor-recipient combinations. Longer-term graft outcome was less impressive. Importantly, DST was associated with a 10% incidence of anti-HLA antibody generation in recipients. This changed the crossmatch result from negative to positive, so denying many patients the potential benefits of renal transplantation. The reduction in frequency of acute rejection episodes that was achieved with DST became insignificant following the introduction of more effective immunosuppressive therapies such as CsA. Further work has suggested that DST in the case of donors who possess maternal HLA antigens not inherited by the recipient reduces the incidence of acute transplant rejection (Burlingham *et al* 1998). It is hypothesised that transfusion leads to reawakening of a T cell memory of neonatal exposure to semiallogeneic maternal cells and soluble (not cell-bound) HLA.

Future strategies to encourage tolerance may involve donor-specific *bone marrow* transfusion. It has been shown in rats (male Lewis kidney donors and female Brown-Norway recipients) that peritransplant infusion of donor bone marrow followed by administration of haematopoietic growth factors (including rh-granulocyte colony stimulating factor (GCSF) and rh-IL-6) reduces the severity of subsequent CAN in inverse proportion to the amount of detectable microchimerism that is produced (Terakura *et al* 1999).

In animal models the systemic administration of CTLA4Ig, a fusion protein that binds B7 molecules on the surface of antigen-presenting cells and therefore blocks the B7-CD28 T-cell *costimulatory* pathway, induces a state of immunotolerance. Azuma *et al* tested the effect of CTLA4Ig on the development of CAN in rats (Azuma *et al* 1996). F344 kidneys were transplanted orthotopically into bilaterally

nephrectomised Lewis recipients. Control animals received low dose CsA for 10 days post-transplantation. In the study groups, a single injection of CTLA4Ig was administered on day 2 post-transplant either alone or in addition to the 10 day CsA regimen. Long-term graft survival was improved in both groups compared to controls. Control animals developed progressive proteinuria and morphological evidence of CAN by 16 to 24 weeks, whereas CTLA4Ig-treated animals did not. These data suggest that T-cell recognition of alloantigen is an important early event in the development of CAN.

Other approaches

Angiopeptin is a long-acting cyclic octapeptide. It has pharmacological actions that closely resemble those of the pituitary hormone somatostatin. *In vitro* studies have demonstrated that it inhibits proliferation and migration of smooth muscle cells and adhesion of mononuclear cells to vascular endothelium. The systemic administration of angiopeptin reduces vascular myointimal thickening in both syngeneic and allogeneic transplant models (Foegh *et al* 1995). There are no reliable data from studies of angiopeptin in human renal transplant recipients with CAN. Human cardiac allograft recipients who received subcutaneous angiopeptin for the first 14 days post-transplantation had less myointimal thickening (as measured by intracoronary ultrasound) than a control group, but clinical outcome data were not reported (Meiser *et al* 1995).

Many other immunological and non-immunological approaches to the treatment of CAN have been considered. These include the use of platelet-activating-factor antagonists (Hayry *et al* 1993), heparinoids (Aziz *et al* 1993), thromboxane receptor antagonists, thromboxane synthase inhibitors and prostacyclin analogues (Teraoka *et*

al 1987), fish oil supplements (Sweny 1993) and 1,25-dihydroxyvitamin D3 (Aschenbrenner *et al* 2001).

Platelet-activating factor (PAF) is a phospholipid mediator of inflammation that is produced by inflammatory cells and endothelial cells. During acute allograft rejection, PAF increases endothelial permeability and recruitment of inflammatory cells within the graft (Renkonen *et al* 1999). It has been shown that a PAF antagonist (WEB 2170) enhances the survival of renal allografts in dogs receiving either a limited course of CsA or a reducing course of CsA and AZA (Watson *et al* 1993). A randomised, double-blind study of the PAF antagonist BN 52021 in *de novo* human cadaveric renal transplant recipients (n = 29) showed a reduced incidence of delayed graft function and acute rejection episodes (Grinyo *et al* 1994). Larger studies with a longer period of follow-up are required.

Heparin inhibits smooth muscle cell migration and proliferation and can reduce the accumulation of extracellular matrix that occurs after arterial injury. Aziz *et al* studied low molecular weight heparin (LMWH) in combination with low dose CsA in a rat heterotopic model of heart transplantation (Lewis-Brown Norway to Lewis). Allograft survival improved significantly in comparison to rats receiving LMWH or CsA alone. In addition, coronary disease affecting the graft was less severe and the histological features of parenchymal rejection were less marked.

Endothelial cell injury induces platelet aggregation and release of thromboxane A2 (TXA₂) and platelet-derived growth factor (PDGF) as well as activating the coagulation cascade. PDGF and TXA₂ are thought to play an important role in the pathogenesis of vascular lesions in chronic vascular rejection (CVR) (Teraoka *et al* 1993). Studies of renal transplant recipients with chronic rejection have shown that

thromboxane synthetase inhibitors and antiplatelet agents may reduce the rate of disease progression, but patient numbers have been relatively small (Teraoka *et al* 1993, Teraoka *et al* 1996).

Aschenbrenner *et al* observed a slower progression of CAN in patients receiving *vitamin D* supplements and proceeded to study control and 1,25-dihydroxy vitamin D-treated rats using a Fisher to Lewis renal allograft model. They reported a reduction in bioactive TGF-beta expression in the latter group but did not assess differences in graft function or graft histology.

Sweny studied the effect of *fish oil supplements* in 14 patients with CAN. It is not clear if biopsy evidence of CAN was obtained in all cases. The method of patient selection is also unclear. Plasma creatinine values ranged from 168 $\mu\text{mol/l}$ to 498 $\mu\text{mol/l}$ with a group mean of 298 $\mu\text{mol/l}$. Renal function was monitored during a 6 month run-in period (reference methods of measuring GFR were not used). MaxEPA (containing polyunsaturated fatty acids and docosahexaenoic acid) was then commenced at a dose of 0.2 ml/kg/day and continued for 6 months. The third phase of the study was a 6 months observation period following cessation of MaxEPA. During the treatment and follow-up periods, the rate of deterioration in graft function was significantly reduced ($p < 0.05$). Urinary protein excretion did not change but there was a large reduction in serum triglyceride levels. Some patients complained of nausea and an unpleasant aftertaste when taking MaxEPA. Larger controlled studies are needed.

Anticipation and prevention of CAN – identification of predisposing factors

5.1 Concepts

5.1.1 TGF-beta 1 gene polymorphisms

As discussed in Section 4.4, expression of endogenous TGF-beta 1 in response to a specific stimulus may vary between individuals as a result of polymorphisms in the promoter, enhancer and leader sequences of the TGF-beta 1 gene. There is emerging evidence of an association between some of these polymorphisms and the early development of CAN (Williams *et al* 2001).

We have examined further the clinical significance of genetic heterogeneity by comparing the frequency distribution of TGF-beta 1 genotypes in renal transplant recipients with stable long-term graft function and those with CAN. Confirmation that genotypic differences are of prognostic importance would lead eventually to tailoring of immunosuppressive regimens guided by the genetic profile of individual transplant recipients for cytokines and other key components of the immune response.

5.1.2 Inter-individual variation in cyclosporin responsiveness using an *in vitro* assay system

In clinical practice it is assumed that the concentration of CsA in the blood is an adequate guide to the degree of CsA-mediated immunosuppressive activity *in vivo*. We have reasoned that an assay of CsA responsiveness at the cellular level might provide a more reliable measure of immunosuppressive activity, leading on to more effective dosing of CsA for individual patients with the potential for an overall improvement in long-term graft outcome.

Previous work with mixed lymphocyte cultures (MLCs, comprising a panel of Epstein-Barr virus transformed, irradiated lymphoblastoid cells that express eight different HLA-DR antigens) has shown that the sensitivity of recipient lymphocytes to prednisolone and CsA *in vitro* predicts the outcome of renal transplantation (Francis *et al* 1988; Francis *et al* 1991). Patients who were 'resistant' to these immunosuppressive agents (defined arbitrarily as a less than 50% inhibition of the MLC response) tended to have higher historical peak panel reactive antibody (PRA) titres but were otherwise no different to 'sensitive' patients, even in respect of the PRA immediately pre-transplant and the number of previous blood transfusions. 'Resistance' to either prednisolone or CsA was associated with an increased incidence of steroid-resistant rejection and immunologic graft loss, and poorer graft function during the first post-transplant year. Similar studies have been performed by Takeuchi *et al*, using Con A as a mitogen (Takeuchi *et al* 1998). It was discovered that interindividual variation in the concentration of CsA and tacrolimus that produced 50% inhibition of *in vitro* lymphocyte blastogenesis (IC₅₀) was much greater in pre-transplant haemodialysis (HD) patients as compared to healthy volunteers, although median values were similar. There was a significant correlation

between sensitivities to CsA and tacrolimus in individual HD patients. Patients who were 'sensitive' to immunosuppression had better graft function and graft survival as compared to 'resistant' patients.

Other applications of *in vitro* testing for transplant recipients have been considered. A bioassay for tacrolimus that is based on the inhibition of alloantigen-induced proliferation of cloned alloreactive T cells has been presented as an alternative to enzyme-linked immunoassay (ELISA) blood level monitoring (Zeevi *et al* 1991). The ELISA may be misleading because it detects biologically inactive drug metabolites as well as parent compound. Other work has shown that a positive culture of lymphocytes from transplant biopsy specimens that are histologically normal predicts a subsequent acute rejection episode and may therefore serve as a guide to dosing of immunosuppressive agents in the early post-transplant period (Weber *et al* 1989).

We have previously performed *in vitro* phytohaemagglutinin (PHA)-driven lymphocyte function tests (using blood samples from healthy volunteers) in the presence of CsA concentrations similar to those measured in the blood of CsA-treated RTRs (Clark *et al* 2000). Abrogation of the PHA-driven response showed wide inter-individual variability with peak inhibition of responsiveness being obtained over a 20-fold concentration range. Moreover, in some cases the degree of inhibition correlated poorly with CsA concentration. Even for individuals in whom a concentration-dependent effect was obtained, maximal levels of inhibition were always below 100% (interindividual range 69% - 96%), signifying that a proportion of stimulated cells are refractory to inhibition by CsA in this assay.

These data suggest that an assay which provides evidence of functional immunosuppression may be useful in guiding the selection of individualised CsA dosing regimens to maximise therapeutic benefit. Our initial approach to this question has been to measure *in vitro* CsA responsiveness in renal transplant recipients with and without CAN, using mononuclear cells extracted from peripheral blood taken prior to the morning dose of CsA (when the blood CsA concentration is at its lowest).

5.1.3 C2 monitoring and the implementation of a newly recommended standard

As discussed in Section 4.1, acute renal transplant rejection may be the strongest risk factor for the development of CAN (Hariharan *et al* 2002). The incidence of acute rejection is likely to be reduced if more reliable ways of ensuring optimal immunosuppression for individual patients are found. One disadvantage of CsA is its poor and variable absorption from the gastro-intestinal tract, leading to unpredictable systemic exposure. The latter can be quantified in terms of the *area under the time-blood CsA concentration curve (AUC)*, which is a more reliable predictor of rejection than trough (pre-dose) blood CsA levels (C_0) (Lindholm *et al* 1993). Exact measurement of AUC is too impractical for routine clinical use, but the more consistent absorption characteristics of the *Neoral*® preparation of CsA has encouraged the use of sparse (abbreviated) sampling techniques. For example, Mahalati *et al* used blood CsA concentrations at 1 (C_1), 2 (C_2) and 3 (C_3) hours post-dose to calculate AUC_{0-4} (the systemic exposure to CsA during the first 4 hours after dosing) in *de novo* renal transplant recipients. A low AUC_{0-4} at 3 days post-transplant predicted the occurrence of an acute rejection episode (Mahalati *et al*

2001). Barama *et al* have reported similar results using AUC0-12 calculated from C0, C2 and C3 (Barama *et al* 2000). When combined with rapid and appropriate *Neoral* dose adjustment, AUC monitoring is associated with very low rates of acute rejection and CsA nephrotoxicity (Clase *et al* 2001).

In the above studies, *the 2 hours post-dose CsA blood level (C2)* was found to be the most reliable single time-point predictor of AUC0-4. C2 may be even more discriminating than AUC because of the importance of high CsA peak concentrations (occurring approximately 2 hours after an oral dose of CsA) in suppressing T cell responsiveness (Halloran 1998). A Cox regression analysis by Keown for the International Neoral Renal Transplantation Study Group showed that freedom from acute rejection depended on CsA absorption efficiency and C2 levels, with a predicted rejection risk of 15-23% for good absorbers (>275 ug/l/mg/kg and C2 >1,500 ng/ml) versus 23-47% for poor absorbers (<200 ug/l/mg/kg and C2 <1,500 ng/ml) (Keown 2001). It has been recommended that a target C2 range of 1,350 - 2,050 ng/ml should be achieved within 5 days of transplantation and maintained during the first post-transplant month, with lower targets for subsequent months (i.e. 1,500 ng/ml in the second month, 1,200 ng/ml in the third and fourth months, 1,000 ng/ml in the fifth and sixth months and 800 ng/ml beyond 6 months). These lower target levels are empirical and need to be tested in the clinical setting.

C2 monitoring has been applied successfully in the fields of heart and liver transplantation (Levy 1998; Cantarovich *et al* 1999). A pilot safety analysis of its use in renal transplant recipients has suggested that CsA dose adjustment based on C2 monitoring has no adverse impact on renal function, at least in the short term, despite a probable net increase in CsA exposure (Cole *et al* 2001). A study by Britto *et al* has

indicated that a lower C2 target may be appropriate for patients who receive antibody therapy at induction (Britto *et al* 2001). The interim results of an international multicentre prospective randomised study of C2 monitoring (MO2ART) have been encouraging both in terms of the safety and efficacy of CsA dosing guided *solely* by C2 values (Buchler *et al* 2002; Pfeffer *et al* 2002).

We have sought to examine the association between C2 levels and early acute rejection in our population of *de novo* renal transplant recipients. We have also compared the sensitivity and specificity of C2 and C0 for identifying patients who develop early acute transplant rejection.

5.1.4 Variability of cyclosporin exposure and its relation to chronic allograft nephropathy

The effect on CsA therapy on late renal allograft survival has been limited in comparison to the substantial improvements in short-term outcome that were seen at the time of its introduction in the early 1980s (Schweitzer *et al* 1991; Thorogood *et al* 1992; Hariharan *et al* 2000). Large studies have found similar rates of graft loss beyond the first post-transplant year for patients receiving prednisolone/CsA and prednisolone/AZA regimens (Beveridge *et al* 1995; Vanrenterghem *et al* 1996). In addition, it has been reported that chronic renal allograft dysfunction (CRAD) secondary to CAN progresses at a similar rate in CsA and non-CsA treated renal transplant recipients (Velosa *et al* 2001). These observations may be explained by CsA having both beneficial and detrimental effects of CsA on transplant survival (including perhaps an effect on patient mortality) that tend to balance out. It is also conceivable that CsA fails to prevent CAN, a major cause of late graft failure for which there are both immunologic and non-immunologic aetiologies (Lewis 1995).

One of the difficulties in resolving these questions is that CAN and chronic CsA nephrotoxicity share common histopathological features and may therefore be indistinguishable. Furthermore, the two processes are not mutually exclusive and often coexist. CsA may even contribute to the development and progression of CAN (Mihatsch *et al* 1995).

Although treatment with CsA has had little effect on late allograft failure in renal transplant *populations*, there is some evidence that for *individual patients* the degree and variability of CsA exposure may be of prognostic importance.

Degree of exposure

Several groups have reported an association between low cumulative CsA dose and CAN (Schroeder *et al* 1995; Citterio *et al* 1998; Opelz 2001). Retrospective data collected by the Collaborative Transplant Study Group indicate that graft survival is also reduced in patients with a high CsA exposure, as measured by the weight-adjusted CsA dose at one year post-transplant (Opelz 2001).

Variability of exposure

In a longitudinal pharmacokinetic study of more than 200 patients by Kahan, estimates of 'area under the blood CsA concentration versus time curve' (AUC) were derived using a multiple sampling technique (Kahan 1998). It was concluded that a greater than 20% coefficient of variation ($[SD/mean] \times 100$) of the dose-corrected, time-averaged blood CsA concentration ($C_{av} = AUC/T$) was an independent risk factor for the development of CAN.

We have performed a case-control study to examine further the contribution of these and other factors to the development of CAN in renal transplant recipients receiving follow-up at St James's University Hospital.

5.2 Laboratory methods

Most of the laboratory work was performed in the Renal Research Laboratories at Manchester Royal Infirmary and the Department of Transplant Immunology at St James's University Hospital in collaboration with Dr PEC Brenchley and Dr B Clark.

5.2.1 Molecular studies

DNA extraction from peripheral blood cells

For each study participant a blood sample was collected into a potassium-EDTA tube and the post-centrifugation cellular sediment retained and stored at -20°C . In the laboratory the sediment was allowed to thaw and the volume adjusted to approximately 7.5 ml by adding phosphate buffer solution (PBS). Lysis Buffer 1 (LB1), consisting of 155mM ammonium chloride, 10mM potassium hydrogen carbonate and 1mM EDTA, was added to make a total volume of 25ml. After mixing and slow rotation of the sample (approximately 30 minutes), the cell nuclei were collected by centrifugation at 2,500 rpm for 10 minutes at room temperature with no brake (IEC Centra 7). The supernatant was removed and 15 ml of LB1 added to wash the pellet. The cycle was repeated and the pellet then vortexed prior to resuspension in 6 ml of Lysis Buffer 2 (LB2), the latter comprising 2% SDS and 25 mM EDTA. Following an incubation period of approximately 2 hours at room temperature, precipitation of protein within the sample was achieved by addition of 2 ml of 10mM ammonium acetate. After vortexing and centrifugation (2,500 rpm for 20 minutes at room temperature, no brake), the supernatant layer was transferred into a fresh tube containing propan-2-ol (isopropanolol) and DNA precipitation was encouraged by

gentle inversion. Centrifugation (2,500 rpm at room temperature for 5 minutes, no brake) was repeated and the supernatant was separated from the DNA pellet by decanting and then micropipetting. The pellet was washed with 3 ml of 70% ethanol, transferred to a microtube to air dry and then resuspended in 500 µl of distilled water. The final DNA concentration of the sample was estimated by spectrometry (260 nM) and a 500 ng/µl solution was prepared for subsequent use.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a laboratory technique that increases the number of copies of a specific region of DNA, thus providing a sufficient quantity of DNA for analysis. Essentially, a DNA preparation is heated to separate its paired double helical strands (*denaturation*). Short 'primer' sequences of synthesised DNA are able to bind to individual strands during cooling (*annealing*). Typically, the 'primers' are an exact match for native DNA sequences flanking a genomic polymorphism that lies in close proximity to the particular gene of interest. The next step is to extend the annealed primer sequences using a DNA polymerase. The polymerase 'reads' the 'sentence' of an opposing native DNA strand and extends the annealed primer's 'sentence' by 'hooking' letters together in a complementary fashion (*extension*). The steps of denaturation, annealing and extension are repeated several times (the DNA polymerase is able to withstand high temperatures) to produce an exponential increase in the number of copies of DNA sequence.

Reaction mixtures were set up in 0.5 ml microfuge tubes. A negative control (water) was included to monitor contamination. A typical reaction mixture consisted of the following:

<i>Component</i>	<i>Stock concentration</i>	<i>Volume</i>	<i>Final concentration</i>
DNA	50 ng/μl	2 μl	5 ng/μl
Reaction buffer	x 10	2 μl	x 1
Primers (x 2)	5 μM	2 μl	0.5 μM
DMSO (dimethylsulphoxide)	100 %	2 μl	10 %
Magnesium chloride	25 mM	variable	2-5 mM
dNTPs (x 4) (deoxyribonucleotides)	2 mM	7.5 μl	0.75 mM
Taq polymerase	5 units/μl	0.1 μl	0.5 units
H ₂ O		to 20 μl	

Four polymorphisms in and around the TGF-beta 1 gene were examined in CsA-treated adult renal transplant recipients with biopsy-proven CAN (n = 41) and ‘controls’ (CsA-treated patients with stable graft function for a minimum of four years post-transplant and a serum creatinine of less than 200 umol/l, n = 57). These are listed below together with a summary of the PCR protocol.

1) **TGF-beta 1 -800** restriction fragment length polymorphism (RFLP)

<i>Step</i>	<i>Temperature</i>	<i>Time</i>
Initial denaturation	94 °C	3 min
Denaturation	94 °C	1 min
Annealing	60 °C	1 min
Extension	72 °C	1 min
Final extension	72 °C	5 min

(32 cycles, PCR product 388 base pairs)

2) **TGF-beta 1 -509 RFLP** (only differences are shown)

<i>Step</i>	<i>Temperature</i>	<i>Time</i>
Annealing	55 °C	1 min

(32 cycles, PCR product 153 base pairs)

3) **TGF-beta 1 codon 10 RFLP** (only differences are shown)

<i>Step</i>	<i>Temperature</i>	<i>Time</i>
Annealing	55 °C	1 min

(35 cycles, PCR product 199 base pairs)

4) **TGF-beta 1 codon 25 RFLP** (only differences are shown)

<i>Step</i>	<i>Temperature</i>	<i>Time</i>
Annealing	62 °C	1 min

(32 cycles, PCR product 297 base pairs)

All of the above reactions were performed using a thermocycler (Perkin-Elmer Ltd, Buckingham, UK). DNA primers were supplied by V H Bio (Newcastle, UK) and deoxyribonucleotides by Gibco Life Technologies (Paisley, UK).

Restriction enzyme digests

The DNA sequence of the PCR products listed above varies between individuals. Restriction enzymes are able to cleave or 'cut' DNA depending on the precise DNA sequence around the cleavage site. Cutting produces shorter fragments of DNA that can be separated and subsequently identified by electrophoresis on an agarose gel. The so-called restriction fragment length polymorphisms (RFLPs) that occur within

the four amplified DNA sequences around the TGF-beta 1 gene are summarised below:

<i>RFLP</i>	<i>Allele frequency</i>	<i>Enzyme</i>	<i>Temperature</i>	<i>Digest</i>	<i>Fragments</i>
TGF-beta 1 -800	0.912/0.088	TaeI	65 °C	3 hours	182 bp/ 206 bp
TGF- beta 1 -509	0.657/0.343	Bsu36I	37 °C	3 hours	36 bp/ 117 bp
TGF- beta 1 codon 10	0.584/0.416	Not I	37 °C	3 hours	17 bp/ 182 bp
TGF- beta 1 codon 25	0.918/0.082	Bgl I	37 °C	3 hours	37 bp/ 260 bp

bp = base pairs

The typical reaction mixture used for restriction enzyme digests was as follows:

<i>Component</i>	<i>Volume</i>
Restriction enzyme buffer (10 x)	2 µl
Bovine Serum Albumin 0.1% w/v solution	0.2 µl
Restriction enzyme	0.2-0.5 µl at 10 U/µl
dH ₂ O	to 5 µl
PCR product	15 µl

Restriction enzyme preparations were supplied by Promega (Southampton, UK). The DNA products of restriction digests were separated by agarose gel electrophoresis. A standard 'ladder' comprising different lengths of DNA fragments was separated on the same gel to aid characterisation of the digest products.

Genotype and allele frequencies in the two patient groups were compared using Chi square.

5.2.2 Lymphocyte responsiveness to cyclosporin *in vitro*

PHA-driven lymphocyte responsiveness *in vitro* was measured in renal transplant recipients with chronic renal allograft dysfunction secondary to CAN (n = 19) and CsA-treated controls with stable graft function (serum creatinine < 200 $\mu\text{mol/l}$) for a minimum of 4 years post-transplant (n = 18). The assay comprised incubation of peripheral blood mononuclear cells (obtained from pre-CsA dose blood samples) with PHA (5 $\mu\text{g/ml}$) in the presence of added CsA (Neoral) at concentrations ranging from 0 - 500 ng/ml. Cultures were pulsed with ^3H thymidine at 72 hours and harvested 18 hours later. The amount of radioactive signal at this time provided an estimate of residual T cell activity (Rackbeta, Pharmacia, UK).

5.2.3 C2 monitoring and acute renal transplant rejection

For a period of 15 months starting in June 2000, trough (C0) and 2 hours post-dose (C2) whole blood CsA concentrations were measured in samples obtained from consecutive renal transplant recipients at or around the fifth post-operative day (monoclonal antibody assay, Abbott Laboratories, Delkenheim, Germany). The standard immunosuppressive regimen comprised prednisolone, AZA and CsA, but

some patients (n = 18) received basiliximab (Simulect) and/ or MMF. In all patients, CsA dose adjustments were made *solely on the basis of regular C0 measurements*.

We examined retrospectively the relationship between C2 levels at or around the fifth post-operative day (day 4 or day 6 if there was no sample on day 5) and the occurrence of acute rejection (AR, biopsy-proven or clinically suspected with response to an increase in immunotherapy) within the first 20 days following transplantation.

Two types of analysis were performed. In the first, a C2 value of $\geq 1,500$ ng/ml at or around day 5 was taken as the standard. Patients were divided into 2 groups according to whether or not this had been achieved. The proportions of patients in each group who developed AR were compared using Chi-square. C0 values for patients with and without AR were compared using Mann-Whitney.

A Bayesian methodology was adopted in the second analysis, thus avoiding the need for arbitrary grouping of patients to test a specific null hypothesis. Bayes' theorem can be helpful in assessing the ability of a diagnostic test to predict a particular clinical outcome in terms of its sensitivity, specificity, likelihood ratio, odds ratio and predictive value. Some of this information is summarised graphically in a receiver operating characteristics (ROC) curve. The ROC curve is a plot of sensitivity against (1 – specificity) and is helpful in selecting a 'cut-off' value that determines whether a test result is positive or negative. In the context of this study, a Bayesian approach allowed us to assess the usefulness of C0 and C2 as predictors of early AR and to evaluate *de novo* the most appropriate 'cut-off' value for C2 in our study population. Thus the sensitivity (the true positive rate) and specificity (the true negative rate) of C0 and C2 at or around day 5 for predicting AR within 20 days of transplantation

were calculated for a range of 'cut-off' values (C0: 200 – 550 ng/ml, C2: 900 – 1,500 ng/ml). C2 concentrations of more than 1,500 ng/ml exceeded the upper limit of the standard curve of the assay. These were reported as '> 1,500 ng/ml' (double dilution was not performed).

5.2.4 Variability of cyclosporin exposure

The renal unit electronic database at SJUH was interrogated to identify adult CsA-treated renal transplant recipients with chronic progressive renal allograft dysfunction (defined as a negatively sloping ROCT plot over a minimum of 6 months) secondary to biopsy-proven CAN (Banff category V, group A) and CsA-treated controls with stable graft function for a minimum of four years post-transplant and a serum creatinine of less than 200 $\mu\text{mol/l}$ (group B). Exclusion criteria included known non-compliance with immunosuppressive medication, hepatobiliary disease and concomitant use of medications that interfere with CsA metabolism. The following variables were recorded when available: age at transplant, gender, years post-transplant, donor source, donor age, donor kidney preservation time, HLA match, occurrence of delayed graft function, immunosuppressive regimen, weight-adjusted CsA dose in the previous 12 months (6 categories; lowest category < 2mg/kg, highest category > 4mg/kg) and coefficient of variation ($C_{\text{var}}C_0$) of dose-adjusted CsA trough blood levels (C_0/dose). Patients transplanted before 1995 had received *Sandimmun* prior to *Neoral*® conversion. The average duration of *Sandimmun* treatment was the same in both groups (median of less than 1 year). CsA data from this period were not considered in view of the likelihood that $C_{\text{var}}C_0$ would be different for the two CsA formulations. CsA data

from the first 3 months post-transplant were also discounted because of uncertainty as to the exact timing of the many CsA dose alterations that were made during this period. $CvarC_0$ was calculated using the following formula:

$$CvarC_0 = (SD[C_0/dose] / \text{mean}[C_0/dose]) \times 100$$

The Chi square test was used to compare categorical variables in the two groups. Continuous variables were compared using the Mann-Whitney and 2-tailed unpaired t-tests as appropriate. In addition, a binary logistic regression analysis was performed using a forward conditional sequential (stepwise) method of entering study variables into a computerised model (SPSS software package version 9.0 for Windows 95, Chicago, USA).

5.3 Results and discussion

5.3.1 TGF-beta 1 gene polymorphisms

The TGF-beta 1 genotype of 41 patients with CAN and 57 CsA-treated controls with stable graft function (as defined previously) was determined. Demographic data for patients in the CAN group are summarised in Section 7.2. Data for 'control' patients are summarised in Table 4 (group B, Section 5.3.4). Although the number of control patients in each of the comparative studies that are described in this chapter differs slightly (due to a failure to collect blood and/or urine samples in some cases), Table 8 can be regarded as a general summary of control group demographics.

Agarose gel DNA electrophoresis was used to confirm DNA amplification by PCR and identify DNA products of the restriction enzyme digests. A selection of gel photographs is shown in Figures 3 - 10. Tables 1 and 2 summarise the allele and genotype frequencies in the two study groups and in the general population (Cambien *et al* 1996) for the four TGF-beta 1 gene polymorphisms (RFLPs) that are listed in Section 5.2.1. It can be seen that none of the inter-group differences in allele or genotype frequency were statistically significant, suggesting that recipient TGF-beta 1 gene polymorphisms do not predict the development of CAN.

It is important to acknowledge the possibility of a type II statistical error in view of the relatively small number of patients in the CAN group. Larger studies are required to address this question.

There may be other possible explanations for our negative findings. Firstly, we did not consider *donor* TGF-beta 1 genotype as this information was generally

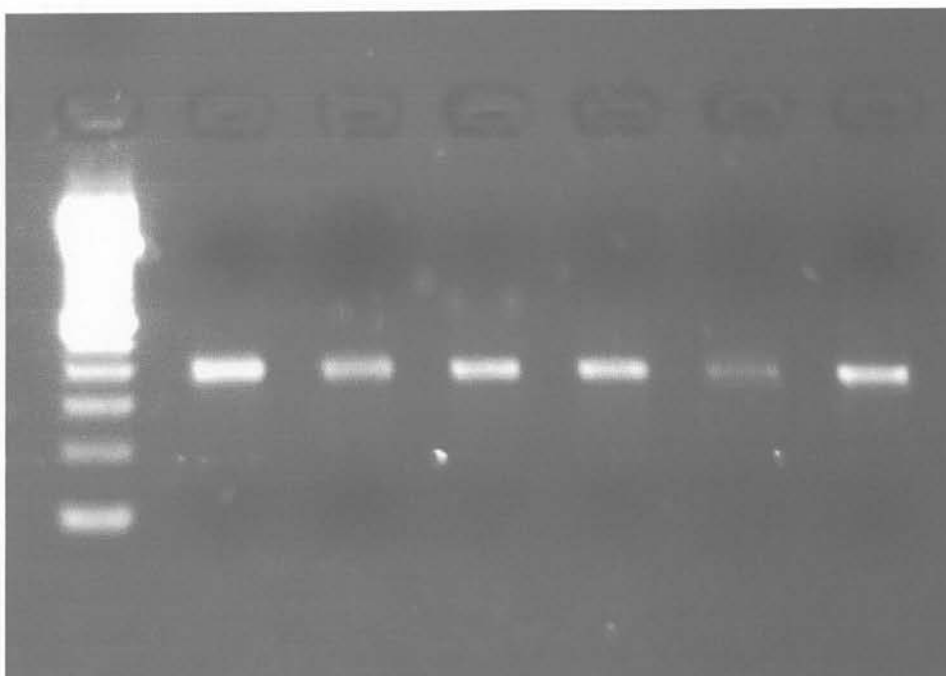


Figure 3. PCR product (400 bp) for the -800 TGF-beta 1 gene polymorphism

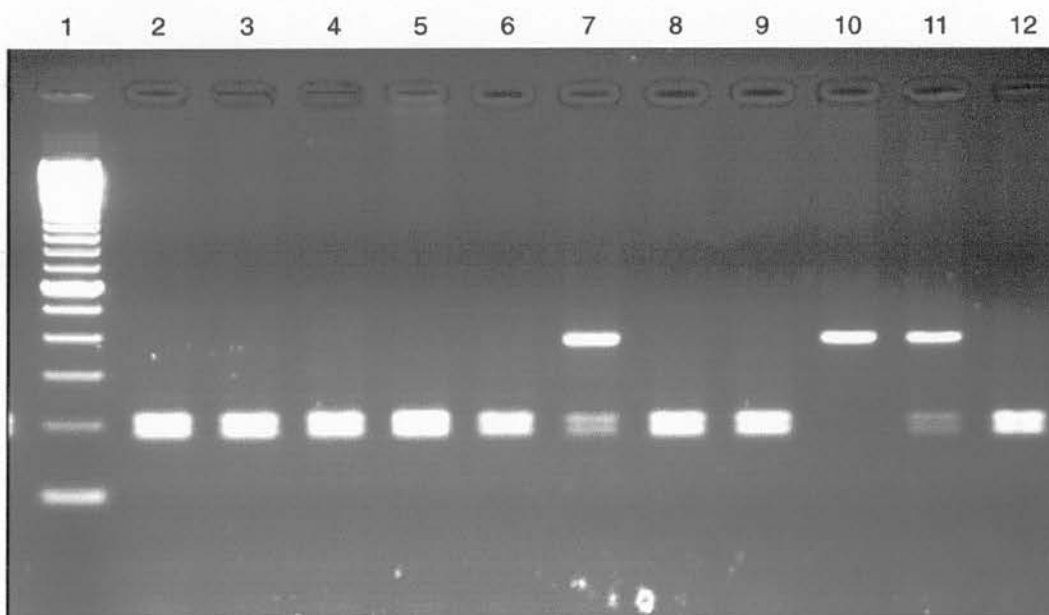


Figure 4. Restriction enzyme digest for the -800 TGF-beta 1 gene polymorphism

Lane 1 - DNA ladder,
Lane 2 - GG homozygote,

Lane 7 - GA heterozygote,
Lane 10 - AA homozygote

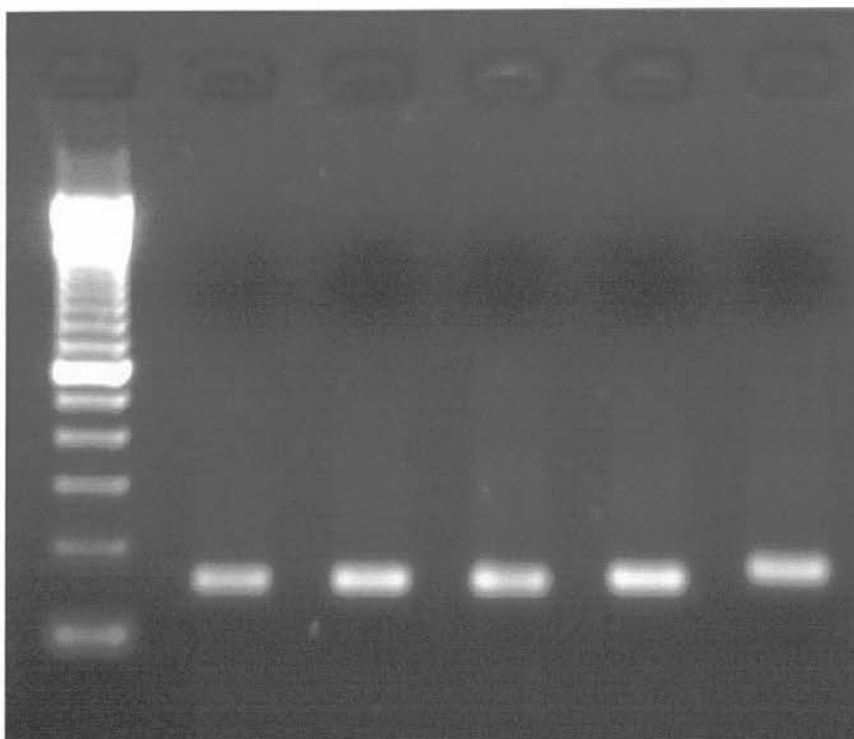


Figure 5. PCR product (153 bp) for the -509 TGF-beta 1 gene polymorphism

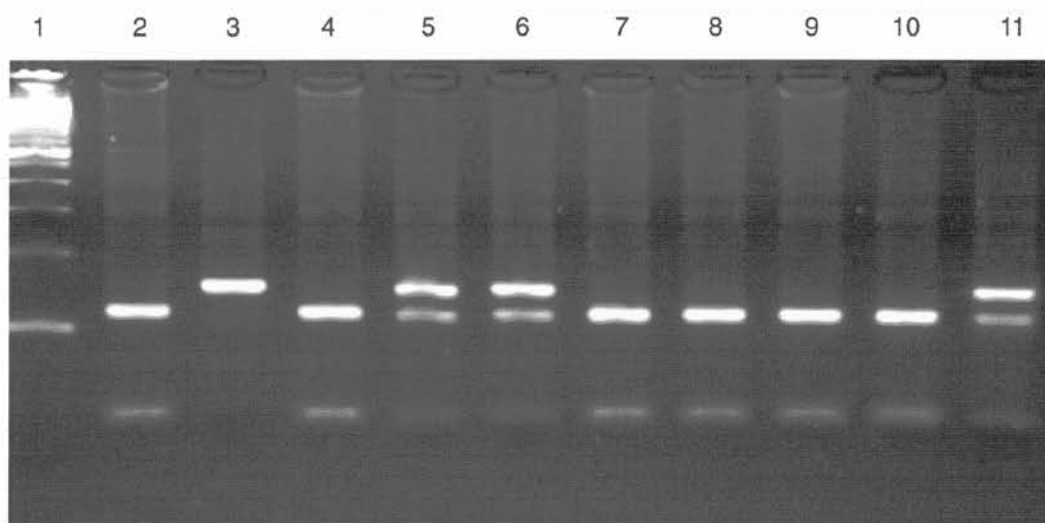


Figure 6. Restriction enzyme digest for the -509 TGF-beta 1 gene polymorphism

Lane 1 - DNA ladder,
Lane 2 - CC homozygote,

Lane 3 - TT homozygote,
Lane 5 - CT heterozygote

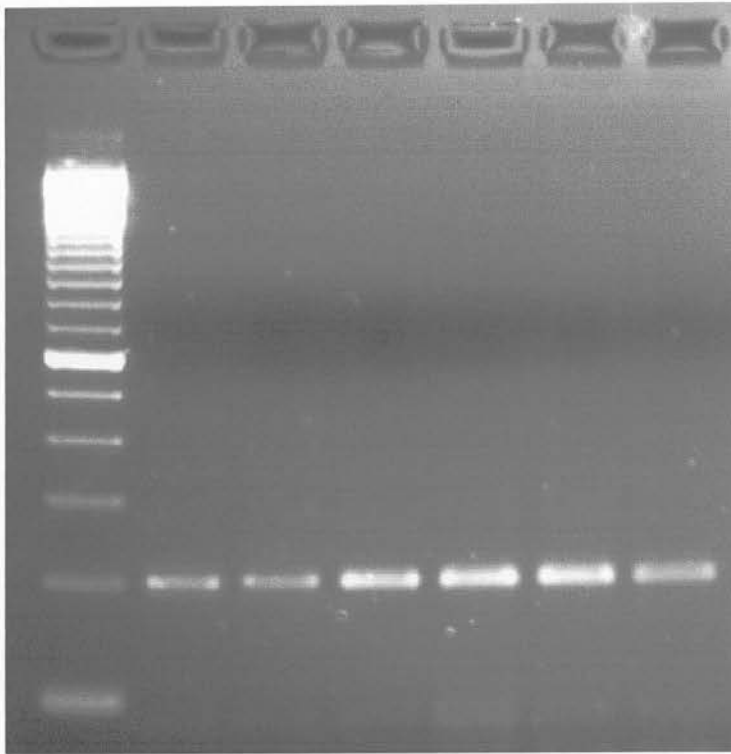


Figure 7. PCR product (199 bp) for the codon 10 TGF-beta 1 gene polymorphism

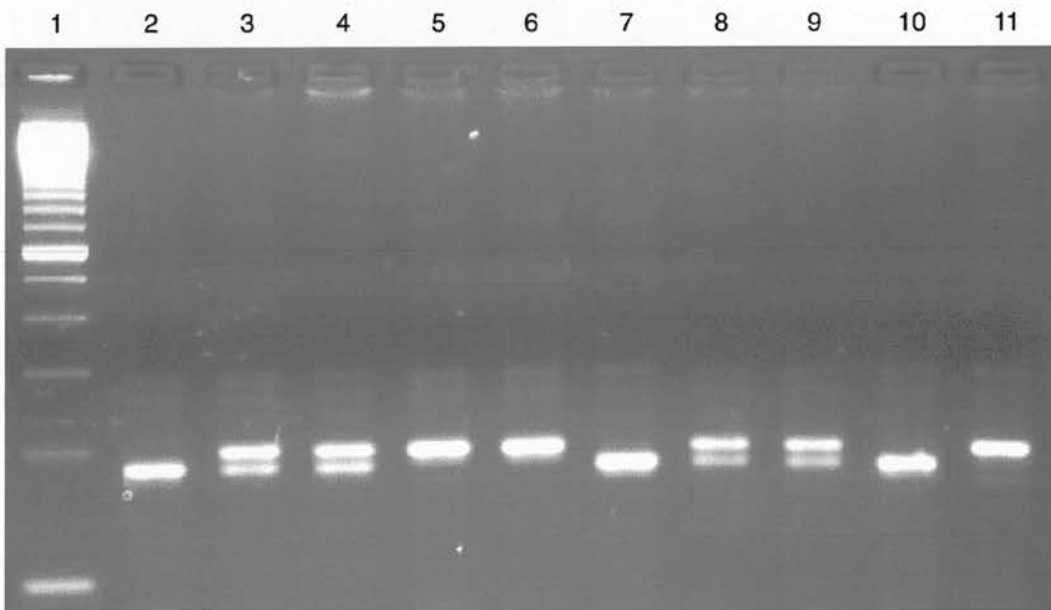


Figure 8. Restriction enzyme digest for the codon 10 TGF-beta 1 gene polymorphism

Lane 1 - DNA ladder,
Lane 2 - CC homozygote,

Lane 3 - CT heterozygote,
Lane 5 - TT homozygote

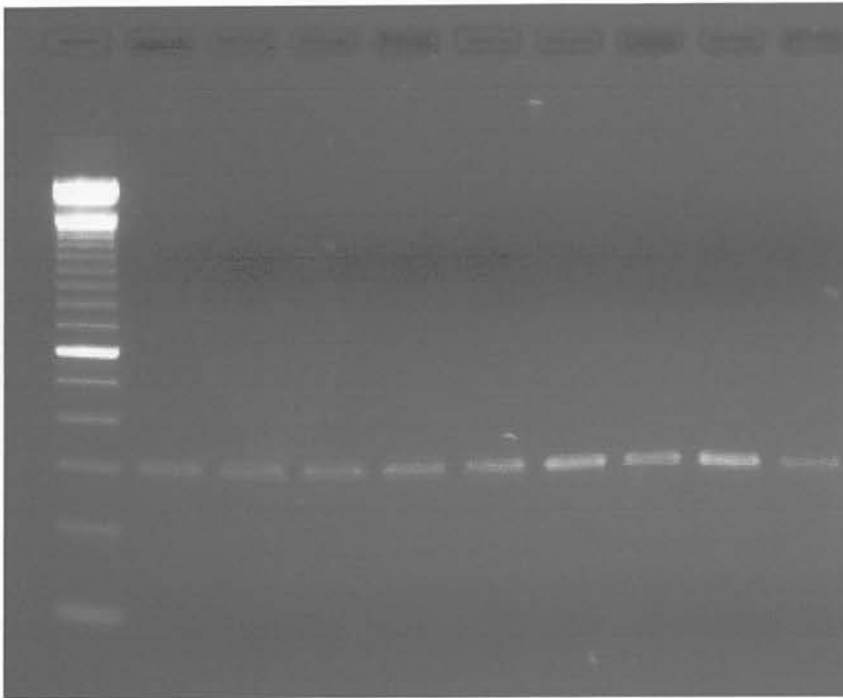


Figure 9. PCR product (297 bp) for the codon 25 TGF-beta 1 gene polymorphism

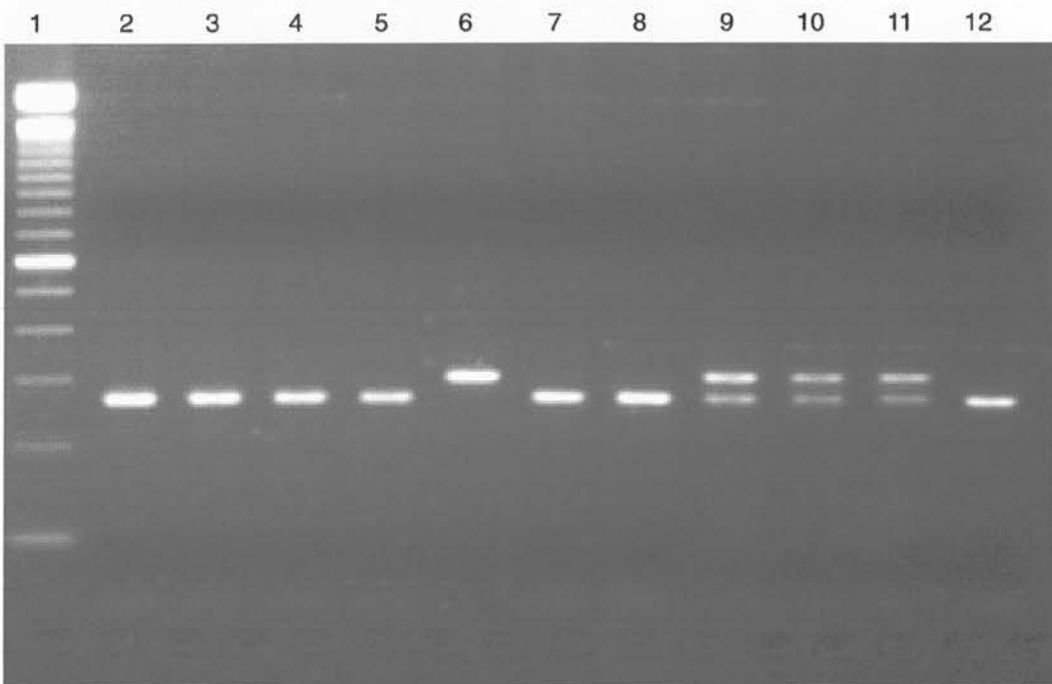


Figure 10. Restriction enzyme digest for the codon 25 TGF-beta 1 gene polymorphism

Lane 1 - DNA ladder, Lane 6 - CC homozygote,
Lane 2 - TT homozygote, Lane 9 - CT heterozygote

unavailable. Evidence from previous studies suggests that donor genotype has an effect on transplant outcome (Sankaran *et al* 1999). Secondly, there may have been a failure to discriminate between chronic CsA nephrotoxicity and CAN on transplant biopsy specimens (see Section 4.5), resulting in a heterogeneous study population. Similarly, there may have been heterogeneity in the control group. The addition of histopathological criteria to those specified in our study would reduce the likelihood of including patients with subclinical CAN, however like most UK transplant centres the Leeds unit does not routinely perform transplant biopsies in patients whose graft function is stable.

Table 1. Allele frequencies for TGF-beta 1 gene RFLPs

RFLP	Allele frequency	Patients with CAN	Patients with stable graft function	General population
-800	G	90 %	93 %	92 %
	A	10 %	7 %	8 %
-509	C	70 %	66 %	65 %
	T	30 %	34 %	35 %
Codon 10	C	44 %	39 %	38 %
	T	56 %	61 %	62 %
Codon 25	G	90 %	95 %	91 %
	C	10 %	5 %	9 %

Table 2. Genotype frequencies for TGF-beta 1 gene RFLPs

RFLP	Genotype frequency	Patients with CAN	Patients with stable graft function	General population
-800	GG	80 %	88 %	85 %
	GA	20 %	11 %	14 %
	AA	0	1 %	1 %
-509	CC	49 %	45 %	42 %
	CT	41 %	42 %	47%
	TT	10 %	13 %	11 %
Codon 10	CC	24 %	19 %	17 %
	CT	39 %	39 %	47 %
	TT	37 %	42 %	36%
Codon 25	GG	83 %	91 %	87 %
	GC	15 %	7 %	12 %
	CC	2 %	2 %	1 %

Another possibility is that some of the patients in the study developed CAN as a consequence of multiple factors including unrecognised poor compliance with immunosuppressive medication, leading to an underestimation of the genetic component. Consideration of a single cytokine genotype in isolation is undoubtedly over-simplistic, but assessment of combinations of genotypes would require a considerably larger study population. Finally, interindividual variation in TGF-beta 1 expression may be of lesser significance in chronic rejection as compared to acute rejection (Sankaran *et al* 1999).

5.3.2 Lymphocyte responsiveness to cyclosporin *in vitro*

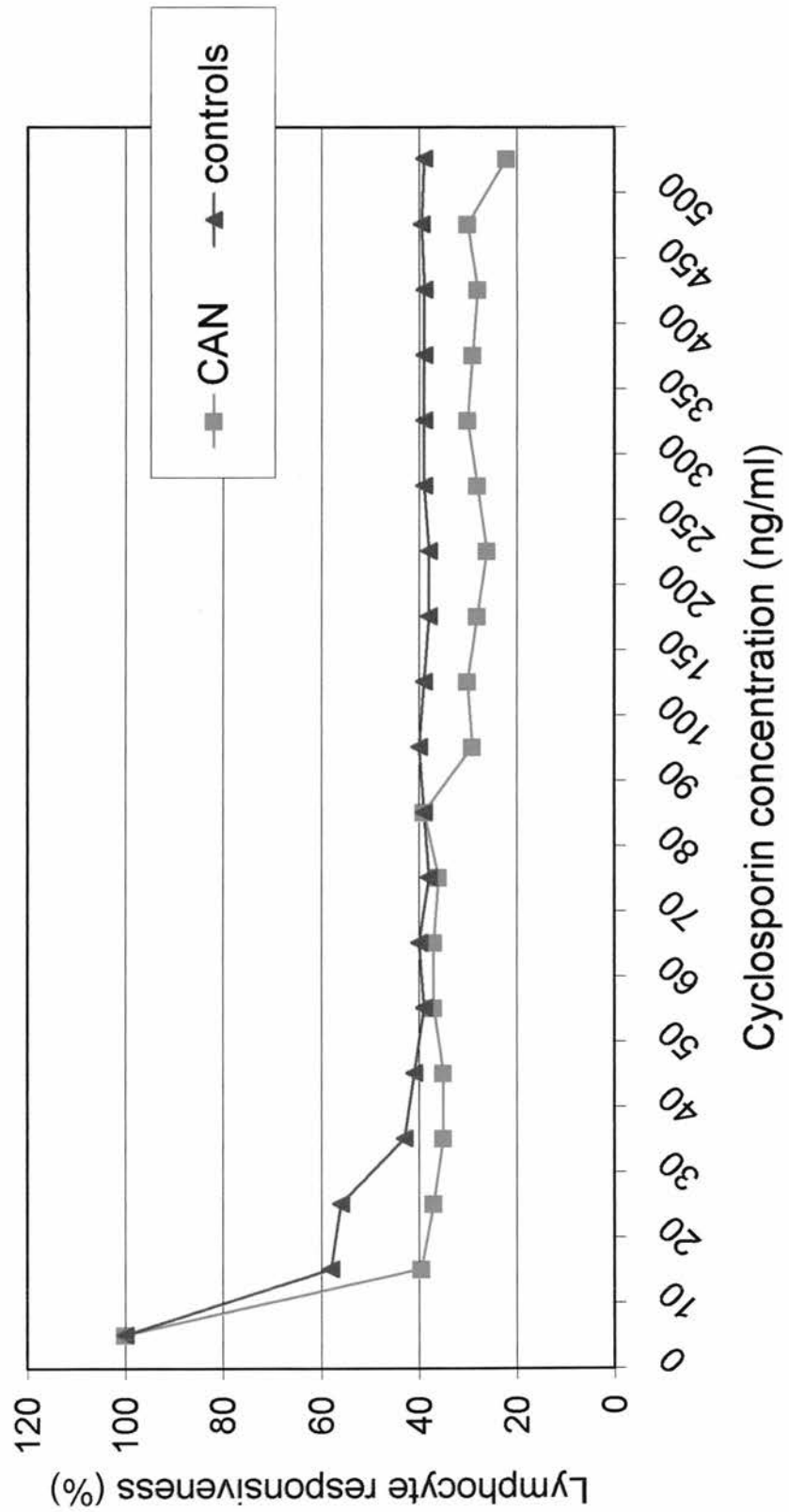
The pre-dose concentrations of CsA in assay blood samples were similar in renal transplant recipients with and without CAN. The mean percentage reduction in lymphocyte responsiveness (LR) *relative to the culture with no added CsA* was greater in patients with CAN at all CsA concentrations, although the difference was not statistically significant ($p = 0.12$, Figure 11).

The similarity of the results in the two groups is clearly not what would be expected from a test that is capable of predicting long-term renal transplant outcome. This may be taken as further evidence of the importance of *non-immunological* factors in the development of CAN. It could be also that a test based on mitogenic stimuli such as PHA is less informative than a test based on a donor-specific cognate alloantigenic stimulus.

There are other ways of interpreting the data. The larger reduction in LR seen in patients with CAN may indicate that they are more sensitive to other (non-immunological) effects of CsA, including perhaps programmed cell death (apoptosis) and tissue fibrosis (both of which are thought to be relevant to the development of CAN). Alternatively, differences in LR reduction could be explained by there being a CsA-mediated immunosuppressive activity *in vivo* which is further from its maximum (i.e. there is greater potential for reducing LR *in vitro*) in patients who develop CAN.

The best way of exploring these concepts further is to perform a large, prospective pre-transplant study of *in vitro* sensitivity to all of the immunosuppressive agents that are in common use, employing both mitogenic and cognate alloantigenic stimuli. This would make it possible to measure absolute reductions in LR rather than change

Figure 11. *In vitro* lymphocyte responsiveness in patients with and without CAN



in LR relative to a pre-established *in vivo* CsA activity (although the effect of the latter was minimised in this study by using pre-CsA dose blood samples).

5.3.3 C2 monitoring and acute transplant rejection

88 adult patients were transplanted and received CsA-based immunosuppression during the period of study. C2 data were available in all but 12 patients, none of whom developed AR. The median C2 value for the 11 patients who developed AR was 1,192 (interquartile range 1,008 - 1,286) ng/ml. The median C0 value for the same 11 patients was 389 (interquartile range 365 - 421) ng/ml, compared to 418 (interquartile range 311 - 469) ng/ml for those without AR ($p = 0.8$).

In the first analysis, a C2 measurement of $\geq 1,500$ ng/ml at or around day 5 was taken as the standard. Patients were divided into 2 groups according to whether or not this had been achieved.

Of the 25 patients with $C2 \geq 1,500$ ng/ml (group A), only one developed AR. 5 patients in group A had received basiliximab (Simulect), 2 in combination with MMF. Another patient was taking MMF in place of AZA. Of the 51 patients with $C2 < 1,500$ ng/ml (group B), 10 developed AR. 9 of the 10 had received standard triple therapy (1 received MMF in place of AZA). Of the 41 patients in group B who did not develop AR, 5 had received basiliximab and 6 were taking MMF instead of AZA. This clinically significant difference in the frequency of AR between groups A and B did not reach statistical significance (Chi square, $p = 0.07$).

The Bayesian analysis allowed us to compare the usefulness of C0 and C2 as predictors of early AR and to evaluate *de novo* the most appropriate 'cut-off' value

Figure 12. A receiver operating characteristics curve for C0 and C2 values

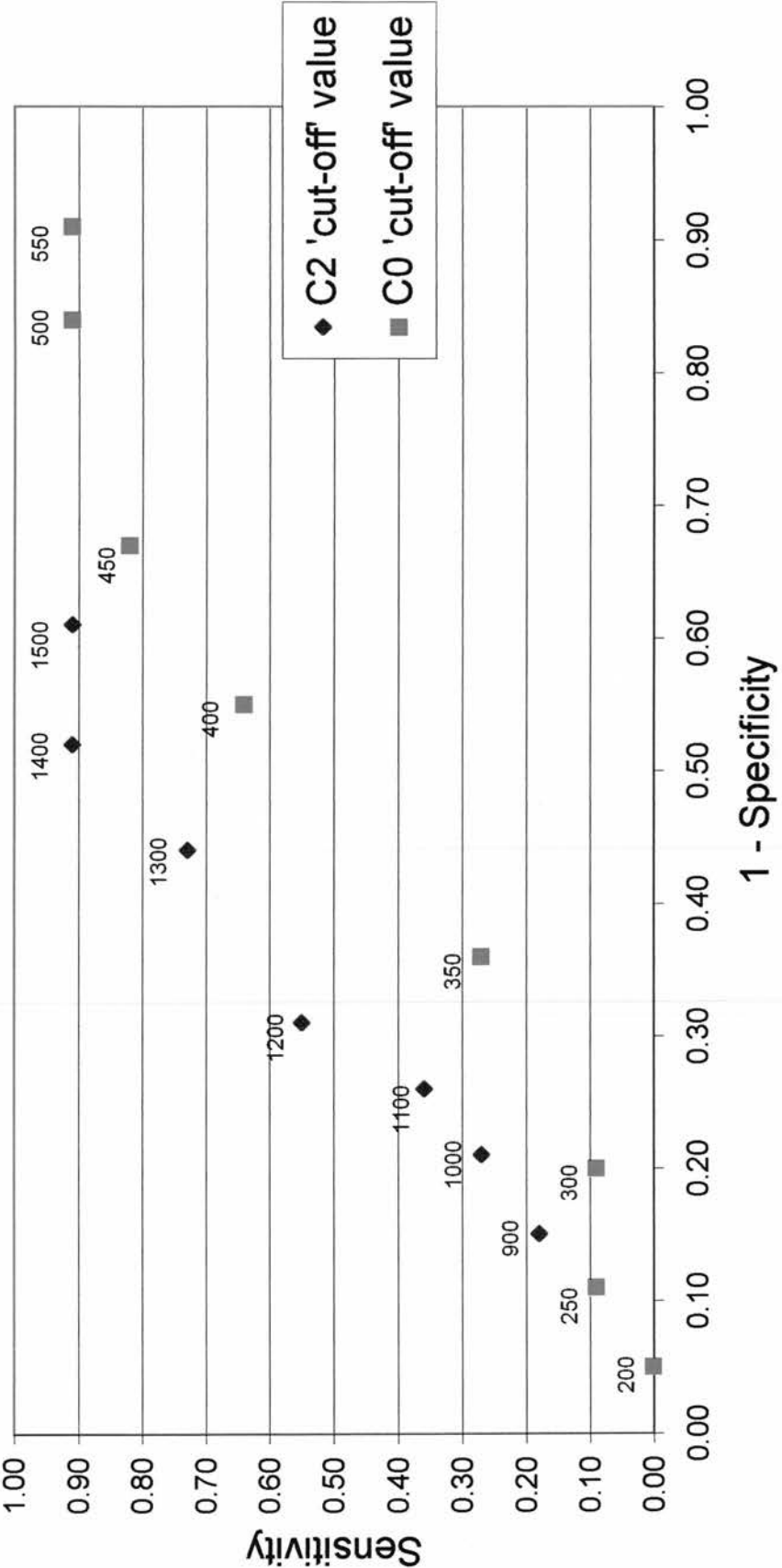


Table 3. Summary statistics for various C2 'cut-off' values

C2 'cut-off' value (ng/mL)	sensitivity	1 - specificity	positive likelihood ratio (PLR ¹)	positive predictive value (PPV ²)	PPV – prior probability of rejection (11/76)
< 900	0.18	0.15	1.20	0.55	0.40
< 1000	0.27	0.21	1.29	0.56	0.42
< 1100	0.36	0.26	1.38	0.58	0.44
< 1200	0.55	0.31	1.77	0.64	0.49
< 1300	0.73	0.44	1.66	0.62	0.48
< 1400	0.91	0.52	1.75	0.64	0.49
< 1500	0.91	0.61	1.49	0.60	0.45

¹ the likelihood of a C2 value below 'cut-off' in patients who develop acute rejection compared to patients who do not develop acute rejection

² the probability of acute rejection in patients with a C2 value below 'cut-off'

for C2 in our study population. Receiver operating characteristic (ROC) plots for C0 and C2 are shown in Figure 12.

It is clear that C2 has greater specificity for early AR than C0 at all levels of sensitivity and is therefore the more discriminating of the two measurements. The positive predictive value (PPV) of C2 for AR at various 'cut-off' levels *in our own population* of transplant recipients is shown in Table 3. The PPV of a test will of course vary according to the incidence of AR in the population that is studied.

This analysis of C2 data collected at or around day 5 post-transplant appears to support the notion that higher peak blood levels of CsA are associated with a lower incidence of early AR, although the sample size is too small to demonstrate statistical

significance and we did not examine rigorously the effect of factors other than CsA exposure that may have influenced graft outcome. There were no major differences in terms of immunosuppressive therapy between the 2 groups. As C2 is a more specific indicator of suboptimal CsA dosing than C0 at any chosen level of sensitivity, the use of a CsA dosing algorithm based on C2 values would be expected to result in fewer unnecessary dose increases during the early post-transplant period as compared to an algorithm based on C0 values.

A pharmacokinetic model for CsA has been developed in conjunction with the Department of Biomedical Sciences at Leeds University, forming the basis for an 'in house' CsA dosing algorithm that is to be applied in the clinical setting.

It remains to be seen if C2 monitoring in combination with rapid and appropriate CsA dose adjustment will be effective in reducing the incidence of early, acute renal transplant rejection. There are resource and logistic implications of a change to C2 monitoring which include additional staffing to ensure accurate timing of venepuncture and increased laboratory costs, and these must be balanced against patient benefit and financial savings that may result from a lower incidence of transplant rejection. In addition, there are currently no prospective data to confirm the findings of retrospective studies that prevention of early acute renal transplant rejection reduces significantly the likelihood of subsequent CAN.

5.3.4 Variability of cyclosporin exposure

Of the 105 patients who met the study inclusion criteria, 3 were excluded because of documented poor compliance with immunosuppressive medication, leaving a study population of 102 patients.

Table 4. Patient demographics according to study group

Variable [number of patients with data]	Group A n = 35	Group B n = 67	P value
Age (years) [102]	31.1 \pm 13.3 ¹	42.0 \pm 16.3	<0.001
Male:female ratio [102]	1.34	1.39	NS
Time post-transplant (years) [102]	6 (4 – 8.5) ²	6 (5 – 9)	NS
HLA mismatches (A/B/DR loci) [94]	0.8/0.8/0.5	0.7/1.1/0.6	NS
Weight-adjusted CsA dose (mg/kg/day) [102]	4.3 \pm 1.6	3.0 \pm 1.6	<0.02
Cold ischaemia time (hours) [83]	15.6 \pm 9.1	16.2 \pm 5.5	NS
Donor age (years) [94]	37.9 \pm 17.6	35.4 \pm 16.5	NS
Donor source (% living donor) [102]	12	4	NS
Delayed graft function (%) [95]	12	17	NS
CvarC ₀ (%) [102]	26.3 \pm 9.4	21.1 \pm 7.6	<0.01
Immunosuppressive regimen (%) [102]			
Pred-Aza-CsA	86	78	NS
Pred-CsA	11	14	
CsA	3	8	
Serum creatinine ³ (umol/l) [102]	230 \pm 73	127 \pm 25	<0.001
C ₀ ⁴ (ng/ml) [102]	138 \pm 45	143 \pm 46	NS

¹ mean \pm SD² median (interquartile range)³ as of June 2001, or the time of immunosuppression conversion if this was sooner (group A patients)⁴ trough blood level of CsA

Data are summarised by patient group in Table 4. The median number of C_0 values per patient was 36 (IQR 27.5 - 43) in RTRs with CAN (group A) compared to 26 (IQR 22 - 33.5) in RTRs with stable graft function (group B, $p = 0.01$). The median number of C_0 values per patient per annum was 6.7 (4.9 - 8.7) in group A and 4.8 (3.7 - 7.3) in group B ($p = 0.005$). Recipient age, $CvarC_0$ and weight-adjusted CsA dose for the preceding 12 months were found to be significantly different in the two groups. As the latter variable is a potentially inaccurate method of standardising CsA dose, an inter-group comparison of body weight was made. The difference was insignificant ($p = 0.36$), suggesting that body weight was not a confounding factor in the analysis.

Low recipient age and a high $CvarC_0$ were the only significant independent predictors of CAN in the multivariate analysis. The regression model coefficients for the two variables are shown in Table 5.

Table 5. Results of the binary logistic regression analysis

Variable	Coefficient	95% Confidence Interval	P value
Recipient age	-0.07	-0.03 to -0.11	<0.001
$CvarC_0$	0.09	0.02 to 0.16	<0.02

This case-control study has demonstrated that there is an increased likelihood of CAN in younger renal transplant recipients and those with variable CsA exposure (as indicated by a high $CvarC_0$), although the difference in mean $CvarC_0$ values for the two study groups was small. There was a trend for patients with CAN to be receiving

a higher weight-adjusted maintenance dose of CsA but this was not a significant predictive variable in the multivariate regression analysis ($p = 0.07$).

Recipient age has been found to predict immunologic graft survival (graft loss secondary to acute or chronic rejection, excluding all other causes including death with a functioning transplant) in previous studies (Pirsch *et al* 1996; Bradley 2000). The explanation for this is unclear. It may be that younger recipients maintain a higher level of immunoresponsiveness following renal transplantation.

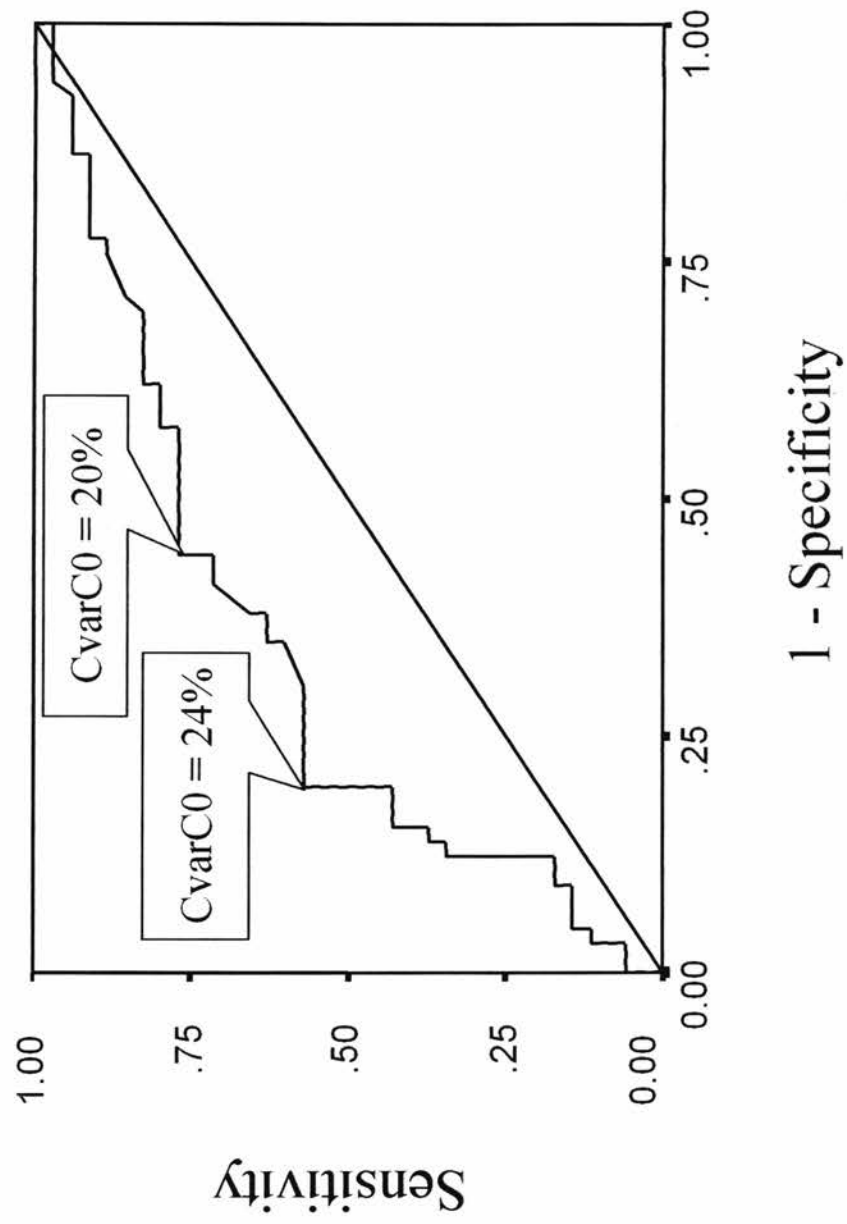
Although a high $C_{var}C_0$ predicts the development of CAN it is apparent that CsA exposure varies considerably even in some patients with apparently stable transplant function. This has been attributed to unpredictable absorption of CsA from the gastro-intestinal tract (Lindholm *et al* 1993) but unrecognised poor compliance with immunosuppressive medication is likely to be another important factor.

As previously discussed, Kahan *et al* used a measure of CsA exposure (C_{av}) that is more accurate than C_0 but less suitable for routine clinical use. Recent studies have shown that ‘sparse sample’ methods (notably measurement of C_2 , the 2-hour post-dose CsA concentration) are reliable alternatives to C_{av} but there remain practical difficulties for outpatient populations (Belitsky *et al* 2000).

The receiver operating characteristics (ROC) curve in Figure 13 shows the sensitivity and specificity of a range of ‘cut-off’ values of $C_{var}C_0$ for predicting the development of CAN in our study population. As a true impression of the variability of CsA exposure requires several C_0 measurements, the reliability of $C_{var}C_0$ is likely to increase with time post-transplant.

The trend towards a higher *weight-adjusted CsA dose* in patients with a diagnosis of CAN contrasts with evidence from previous studies. One explanation for this finding

Figure 13. A receiver operator characteristics curve for CvarC0 values



is a difficulty in discriminating between CAN and chronic CsA toxicity. The histopathological features of chronic CsA toxicity in renal allografts include striped tubulointerstitial fibrosis, tubular atrophy and nodular hyperplasia of afferent arterioles (Feutren *et al* 1992) but only non-specific features are present in some cases. Klintmalm *et al* compared renal biopsy features in both CsA-treated and non-CsA-treated recipients and found more extensive interstitial fibrosis and tubular atrophy in the former group (Klintmalm *et al* 1984). There was a positive correlation between the severity of the histopathological features and both CsA trough levels and the cumulative prescribed CsA dose during the first 6 post-transplant months.

An alternative explanation for the association between CsA exposure and CAN seen in this study is that CsA has effects other than an alteration in renal hemodynamics that are central to the development of CAN. For example, even small doses of CsA promote the synthesis *in vitro* of extracellular matrix components such as collagen type III (Ghiggeri *et al* 1994; Oleggini *et al* 2000). CsA may also stimulate production of TGF-beta 1, a cytokine that has both immunosuppressive and fibrogenic properties (Shihab *et al* 1996). Kahan has reported a positive correlation between serial C2 values and the development of CAN within 3 years post-transplant (Kahan 2002).

The similarity of mean serum C_0 concentrations in the two study groups (138 ± 45 ng/ml in group A vs 143 ± 46 ng/mL in group B, $p = \text{NS}$) presents a third possible explanation, namely that patients who develop CAN may be poorer absorbers of CsA with lower peak blood CsA concentrations. This could be explored by looking at C2 data.

In summary, our data suggest that renal transplant recipients with highly variable CsA exposure have an increased likelihood of developing CAN. Investigation of such patients may allow differentiation between true intrinsic variability of CsA absorption and other factors (previously unrecognised poor compliance with CsA treatment, irregular medication/foods that interfere with CsA metabolism) and so lead on to appropriate interventions.

Non-invasive methods of detecting early CAN

6.1 Concepts

6.1.1 TGF-beta 1 expression in plasma and urine

TGF-beta 1 expression was measured in plasma and urine samples that were provided by patients who participated in the immunosuppression conversion study. The analysis was undertaken for two main reasons. Firstly, there is some doubt as to the activity of TGF-beta 1 at different stages in the development of CAN as there are no prospective longitudinal studies. One group has reported that urinary excretion of TGF-beta 1 increases in proportion to the severity of CAN (Boratynska 1999). Secondly, as discussed in Section 4.4, some authors have claimed that TGF-beta 1 expression is promoted by CsA. It would therefore be of interest to study the pattern of TGF-beta 1 expression in CsA-treated patients with CAN who undergo immunosuppression conversion (CsA to tacrolimus/ addition of MMF with CsA dose reduction).

6.1.2 NAG expression in urine

The lysosomal enzyme N-acetyl β -D glucosaminidase (NAG) is synthesised by proximal renal tubular epithelial cells. If these cells become damaged, NAG leaks into the tubular lumen and is detectable in the urine. It has been shown that low urinary NAG excretion during the immediate post-transplant period predicts

satisfactory early graft function (Matteucci *et al* 1998) and a rise in NAG excretion typically precedes a rise in serum creatinine. Kontanko *et al* studied NAG excretion between days 7 and 28 post-transplant in 33 renal allograft recipients who had an uncomplicated first post-operative month (Kotanko *et al* 1996). Data were evaluated in relation to graft status after 4 and 6 years. A Cox proportional hazards model identified *low* urinary NAG excretion (adjusted according to the urine creatinine concentration) as an independent risk factor for graft loss. This finding is difficult to explain. One theory is that low NAG excretion reflects a low size of graft (the source of NAG) to recipient body mass (a determinant of urinary creatinine excretion) ratio, which is itself known to be associated with poorer long-term graft survival. Other work suggests that NAG excretion is dependent on CsA dose (Marchewka *et al* 1999).

It is not clear whether urinary excretion of NAG is raised in CAN. A study by Wellwood *et al* examined urine NAG excretion in 181 renal allograft recipients over a period of 15 months (Wellwood *et al* 1978). Their finding of high levels of NAG excretion immediately post-transplant with a subsequent reduction in uncomplicated cases is consistent with other studies, but it was also noted that excretion increased in association with chronic rejection, hypotension, renal artery stenosis, renal vein thrombosis and the administration of gentamicin.

We have tested the following hypotheses:-

- (1) The magnitude of urine NAG excretion is proportional to the severity of CAN
- (2) A reduction in urine NAG excretion is an early and reliable non-invasive marker of response to a therapeutic intervention for CAN (immunosuppression conversion in this study).

6.1.3 Serum cystatin C and GFR prediction equations based on serum creatinine

The ability to detect chronic renal allograft dysfunction at an early stage in its development is important in terms of establishing a diagnosis and initiating appropriate treatment. Serum creatinine is used as a measure of renal function in clinical practice, but there are significant disadvantages in terms of its poor sensitivity and the effect of non-renal factors on both the blood concentration of creatinine and the performance of creatinine assays (Perrone *et al* 1992). Creatinine clearance may be calculated from measurements of creatinine in blood and urine. Such estimations are reported to be inaccurate, especially if supervision of timed urine collections is inadequate. Renal tubular secretion of creatinine leads to a significant overestimation of GFR when excretory function is impaired such as in CAN.

More accurate methods for determining GFR have been described, including inulin clearance and clearances of chelated isotopes such as [^{51}Cr] EDTA and [^{125}I] iothalamate. These methods involve the exposure of patients to ionising radiation. They are time-consuming, laborious and expensive and are not used routinely in clinical practice.

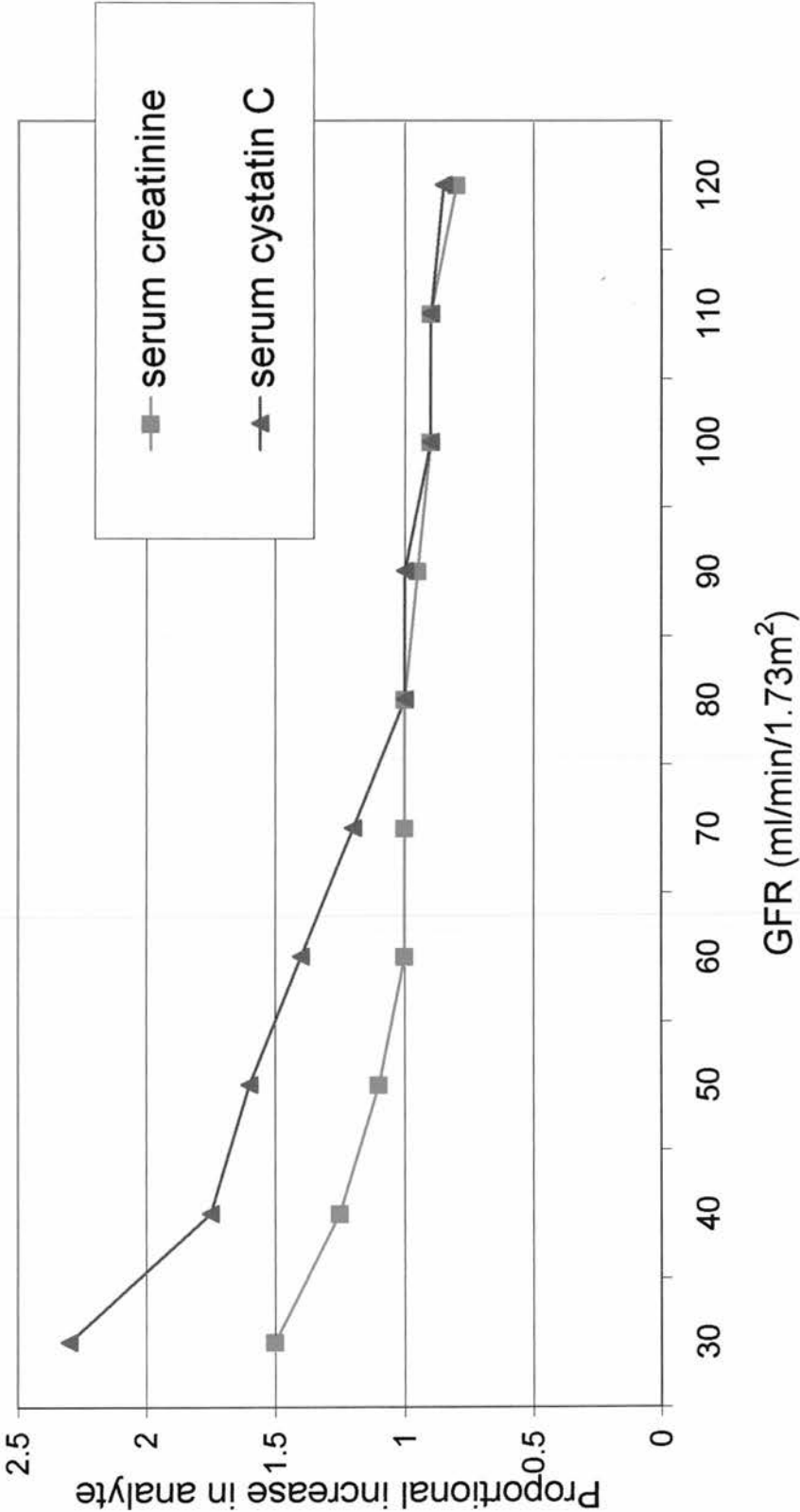
Serum cystatin C

There has been interest in finding simple methods of measuring renal function that are superior to serum creatinine. Cystatin C is a non-glycosylated 13 kD basic protein that inhibits cysteine protease. It is synthesised by all nucleated cells (unlike creatinine) and its rate of production is not influenced by inflammatory conditions (unlike beta-2 microglobulin). Its concentration in the serum is determined mainly by

the glomerular filtration rate (GFR) (Simonsen *et al* 1985; Grubb 1992). Different assay methods for measuring cystatin C have been described, including enzyme amplified single radial immunodiffusion (SRID), ELISA and latex particle enhanced immunoturbidometry (PENIA). The latter is an automated technique and can therefore be used in clinical practice. There is convincing evidence that serum cystatin C is a more sensitive endogenous marker of mild renal dysfunction than serum creatinine (Figure 14, Newman *et al* 1995).

Previous work has suggested that serum cystatin C measurements in *paediatric transplant recipients* tend to be higher than those obtained in children with native renal dysfunction at all levels of renal clearance (Bokenkamp *et al* 1999). This may be explained by the presence of factors that interfere with the cystatin C assay, either directly or *via* metabolic changes induced by immunosuppressive therapy. Other possibilities include tubulo-interstitial damage, leading to a back-leak of intact cystatin C from renal tubules into the circulation, and impaired filtration of cystatin C resulting from increased protein binding within the circulation. There is no experimental evidence to support any of these theories at present, except for a recent study by Risch *et al* that reported an association between higher serum cystatin C concentrations and glucocorticoid therapy in renal transplant recipients (Risch *et al* 2001). The mechanism underlying this association (e.g. change in the rate of cystatin C production and release from cells, interference with cystatin C assays) has not yet been elucidated.

Figure 14. The relationship of serum cystatin C and serum creatinine to GFR (adopted from Newman *et al* 1995)



A study of 33 *adult renal transplant recipients* with stable graft function examined the relationship between GFR estimations obtained from [125 I] iothalamate clearance, creatinine clearance (using the Cockcroft-Gault and Walser equations) and serum cystatin C (Risch *et al* 1999). In terms of positive predictive value and positive likelihood ratio (taking a GFR of less than 60 ml/min/1.73m² as abnormal), serum cystatin C was of similar diagnostic value to calculated creatinine clearance.

Tian *et al* detected cystatin C in the *urine* of patients with native renal impairment (Tian *et al* 1997). The fractional clearance of cystatin C varied inversely with creatinine clearance. It is not clear whether this finding reflects variation in the severity of proximal tubular damage or simply a delivery rate of cystatin C to intact nephron units that exceeds tubular reabsorption capacity.

We have sought to assess the correlation between serum cystatin C (measured using a particle-enhanced nephelometry technique (PENIA)), GFR and serum creatinine measurements in an adult population of renal transplant recipients with CAN. A strong correlation with GFR would establish the validity of cystatin C measurements in this setting.

GFR prediction formulae

An alternative to *measuring* GFR by a reference method is to produce reliable *estimates* of GFR using formulae that are based on biochemical, demographic and anthropometric data. We have compared GFR estimates from such formulae with radioisotope GFR measurements in a population of renal transplant recipients with impaired graft function secondary to CAN. The formulae are summarised below:

Jelliffe equation (Jelliffe 1973)

Creatinine clearance (males) = $98 - 16 [(age\ in\ years - 20) \div 20] \div plasma\ creatinine\ (umol/l)$
(multiply value by 0.9 for females)

Cockcroft-Gault equation (Cockcroft *et al* 1976)

Creatinine clearance (males) = $1.2 \times [(140 - age\ in\ years) \times body\ weight\ (kg)] \div serum\ creatinine\ (umol/l)$
(multiply value by 0.85 for females)

Nankivell 'B' equation (Nankivell *et al* 1995)

GFR = $[6.7 \div serum\ creatinine\ (mmol/l)] + [body\ weight\ (kg) \div 4] - [urea\ (mmol/l) \div 2] - [100 \div height^2\ (m^2)] + [35\ for\ males, 25\ for\ females]$

Walser equation (Walser *et al* 1993)

$GFR \times 3 \div height^2\ (m^2) = a + [b \times serum\ creatinine\ (mg/dl)] + [c \times age\ in\ years] + [d \times weight\ in\ kg]$

	males	females
a =	-6.66	-4.81
b =	+7.57	+6.05
c =	-0.103	-0.08
d =	+0.096	+0.08

MDRD (Modified Diet in Renal Disease) equation (Levey *et al* 1999)

$GFR = 170 \times [PCr]^{-0.999} \times [age]^{-0.176} \times [0.762\ if\ patient\ female] \times [1.180\ if\ patient\ black] \times [SUN]^{-0.170} \times [Alb]^{+0.318}$

PCr = serum creatinine concentration (mg/dl), SUN = serum urea nitrogen concentration (mg/dl), Alb = serum albumin concentration (g/dl)

Bokenkamp formula for cystatin C (Bokenkamp *et al* 1999)

$$\text{GFR}_{\text{cys}} = [137 \div \text{cystatin C (mg/l)}] - 20.4$$

The latter four formulae predict GFR rather than creatinine clearance, which exceeds the GFR measurement in patients with renal dysfunction. The *MDRD formula* has been derived and validated using [^{125}I] iothalamate as the reference method in large cohorts of patients with native renal impairment ('training sample', $n = 1,070$; 'validation sample', $n = 558$, mean GFR 40 ± 20 ml/min/1.73m²) (Levey *et al* 1999). A preliminary study by Bedros *et al* has suggested that the formula provides reliable estimates of graft function in renal transplant recipients (Bedros *et al* 1998). As the only measured inputs required for the formula are results of routine laboratory tests, GFR values can be generated by a computerised reporting system with access to these data. The *Walser formula* was derived from patients with a low renal clearance and has been shown previously to provide a reliable estimate of GFR in renal allograft recipients (Walser *et al* 1993; Goerdts *et al* 1997). The *Nankivell formula* was derived from a population of renal transplant recipients (Nankivell *et al* 1995). The Bokenkamp formula was derived from children (mean age 11.2 ± 4.8 years) with a wide range of renal disease. The concentration of cystatin C in the serum is reported to be independent of lean body mass, body surface area (hence the absence of anthropometric terms in the formula) and age beyond one year.

6.2 Laboratory methods

Most of the laboratory work was performed in the Renal Research Laboratories at Manchester Royal Infirmary and the Department of Transplant Immunology at St James's University Hospital in collaboration with Dr PEC Brenchley and Dr B Clark.

6.2.1 Enzyme-linked immunosorbent assay (ELISA) for TGF-beta 1 in plasma and urine samples

Blood samples were collected into EDTA tubes and centrifuged at 3,000 rpm for 10 minutes at 4 °C with medium brake. 1 ml aliquots of plasma were transferred to cryovials and stored in cryoboxes at -70 °C. Urine samples were collected into plain Universal tubes and then transferred to cryovials for storage alongside the plasma samples.

The ELISA protocol has been described elsewhere (Coupes *et al* 1994). Essentially, the wells of a MicroFLUOR 'W' plate (Dynatech Technologies, Chantilly, USA) were coated with 100 µl of a solution comprising 2.5 µg/ml mouse monoclonal antibody to TGF-beta 1, 2 and 3 (Genzyme, Cambridge, USA) in coating buffer and left overnight in a damp box at 4 °C.

The coating solution was then removed by flicking and the wells were 'blocked' with 150 µl of ELISA buffer (EB, a 0.1% w/v solution of bovine serum albumin (BSA, First Link Ltd, Brierley Hill, UK) in PBS). Meanwhile, a standard curve of recombinant human TGF-beta 1 (rhTGF-beta 1, R and D Systems, Abingdon, UK) solutions was produced by double dilution from 5 ng/ml to 0.075 ng/ml. A dilution

of the internal standard (NIBSAC TGF-beta 1, 1.6 ng/ml, National Institute of Biostandards and Controls, UK) was also prepared.

After completion of the blocking step (1-2 hours), the plate was washed 5 times in washing buffer (PBS and polyoxyethylene sorbitan monolaurate (Tween)). 100 µl of the standards and NIBSAC TGF-beta 1 were pipetted into wells in duplicate. Samples were also pipetted in duplicate and diluted to a volume of 100 µl with BSA (plasma 1:4, urine 1:1). The plate was then left overnight in a damp box at 4°C.

After further washing, 100 µl aliquots of 2 µg/ml chicken anti-TGF-beta 1 (R & D Systems) were applied to each well. The plate was then left for 2 hours at room temperature on a plate shaker. Another washing step was performed and 100 µl of 1:100,000 peroxidase-conjugated donkey anti-chicken antibody (Jackson ImmunoResearch Laboratories, Luton, UK) were added to each well. After incubation at room temperature for 1 hour, washing was repeated and 100 µl of chemiluminescent signal substrate comprising equal volumes of peroxide and luminol enhancer solutions (SuperSignal® West Pico, Pierce, USA) were applied to each well. The plate was shaken for 2 minutes prior to reading of signal with a luminometer (Microumat, EG & G Wallace, Milton Keynes, UK). The results were analysed using Mikrotek software.

The assay is capable of measuring TGF-beta 1 concentrations in excess of 75 pg/ml with a working range of up to 25,000 pg/ml. The intra-assay coefficient of variation is approximately 5%. The detection antibody has been shown to be specific for TGF-beta 1. Neither freeze thawing or prolonged incubation of blood or plasma at room temperature alters the detection of TGF-beta 1. There is no detectable TGF-beta 1 in normal plasma.

Measurements of TGF-beta 1 in urine were standardised by calculating TGF-beta 1: urine creatinine ratios. Urine creatinine was measured using an 'in-house' multiwell plate version of the Sigma creatinine assay (Sigma Diagnostics, Gillingham, UK). Essentially, urine samples were diluted to a 1 in 10 concentration using distilled water. Sequential dilutions of the Sigma Standard Creatinine Solution (15 mg/dl) were prepared. 20 μ l aliquots of the samples and the creatinine dilutions were pipetted onto an assay plate. 200 μ l of an alkaline picrate solution were added and mixed on a plate shaker. The absorbance for each well was read at a wavelength of 490 nm. 7 μ l of Sigma Acid Reagent was then added to all wells and the plate was read at 490 nm after 5 minutes. The creatinine concentration of each sample was calculated from the difference between the two readings.

Plasma and urine samples were collected from patients with CAN who were participating in the immunosuppression conversion study (Chapter 7) on a monthly basis. Samples from CsA-treated 'control' patients with stable long-term graft function (serum creatinine < 200 μ mol/l) for a minimum of 4 years post-transplant were also collected. Both the study and control patients were identified by interrogation of the electronic record of patients receiving transplant follow-up care at St James's University Hospital in Leeds. Differences between groups were compared using Mann Whitney and the Kruskal-Wallis test for independent samples.

6.2.2 Measurement of NAG in urine

Morning urine samples were obtained from patients with CAN ($n = 25$, mean serum creatinine 225 μ mol/l) and a number of CsA-treated controls with stable graft function for a minimum of 4 years post-transplant and a serum creatinine of < 200

umol/l ($n = 58$, mean serum creatinine 125 umol/l). Samples were collected into plain Universal containers and transferred to the Department of Clinical Pathology at St James's University Hospital, where the urine was pipetted into polypropylene tubes and stored at -20°C pending analysis. For patients with CAN, samples were collected weekly for one month and then monthly for 6 months.

A colorimetric assay was used to quantify the amount of NAG in all urine samples (PPR Diagnostics Ltd, London, UK). The test is based on NAG-induced hydrolysis of a substrate releasing a phenolic compound that, in the presence of buffer salts, produces a red colour in proportion to the degree of hydrolysis. The intensity of the coloration can be read using a spectrophotometer set to a wavelength of 505 nm. The assay has been described in more detail elsewhere (Yuen *et al* 1984). NAG measurements were adjusted according to the urine creatinine concentration.

The data were analysed in relation to two key questions. Firstly, baseline measurements of patients with CAN were compared with those of CsA-treated 'control' patients with stable long-term graft function for a minimum of 4 years post-transplant to find evidence of an association between urinary NAG excretion and CAN. Secondly, the effect on NAG excretion of immunosuppression conversion (CsA to tacrolimus/ azathioprine to MMF with CsA dose reduction/ 'no change' controls) and intraindividual variation in transplant function (measured using a reference radioisotopic method) was explored.

6.2.3 Estimates of renal function - GFR, serum cystatin C and GFR prediction equations based on serum creatinine and serum cystatin C

$^{99}\text{mTcDTPA}$ -GFR was measured in 33 CsA-treated patients (10 female, age range 20 to 67 years, body mass index 18 to 32 kg/m^2) with CAN. Calculated GFR values

were obtained from the MDRD, Jelliffe, Cockcroft and Gault, Nankivell 'B', Walser and Bokenkamp formulae using data collected on the day of the isotope study. These were compared with $^{99m}\text{TcDTPA}$ -GFR measurements (corrected for body surface area using the DuBois equation) by calculating bias (the median difference between calculated GFR and $^{99m}\text{TcDTPA}$ GFR) and scatter (the median absolute difference between calculated GFR and $^{99m}\text{TcDTPA}$ GFR).

Serum cystatin C was measured using PENIA (Dade Behring, Marburg, Germany). The principle of the method is that polystyrene particles coated with antibodies to cystatin C agglutinate when mixed with samples containing cystatin C. Agglutination alters the intensity of scattered light within a nephelometer. The concentration of cystatin C in a sample is derived by comparison with nephelometer readings obtained using standard cystatin C solutions of known concentration. The assay has been described in more detail elsewhere (Finney *et al* 1997). Tests of correlation between $1/\text{cystatin C}$, $1/\text{serum creatinine}$ and $^{99m}\text{TcDTPA}$ -GFR data were performed at the start of the study and after 6 months.

6.3 Results and discussion

6.3.1 TGF-beta 1 expression in plasma and urine

There was no detectable TGF-beta 1 expression in *plasma* samples from CAN patients and controls with the exception of a single patient in the CAN group. He subsequently underwent immunosuppression conversion (MMF/ reduced dose CsA, see Chapter 7) and there was a trend of decreasing TGF-beta 1 expression during the period of follow-up.

Figure 15 shows the distribution of adjusted *urine* TGF-beta 1 concentrations in the two study groups. Interestingly, urine TGF-beta 1 excretion was significantly higher in controls as compared to patients with CAN.

Box plots of urine TGF-beta 1 data that were collected over a period of 6 months from patients with CAN are shown according to their randomised treatment intervention (MMF and reduced dose CsA/ tacrolimus in place of CsA/ continuation of CsA-based immunosuppression) in Figure 16. The boxes indicate the upper and lower quartiles and the central line is the median value. The points at the end of the 'whiskers' are the 2.5% and 97.5% values. Median values of urine TGF-beta 1 were fairly constant over time in the control group of patients. It is perhaps surprising in view of the known effect of CsA on TGF-beta 1 expression that urine TGF-beta 1 values did not decrease in patients who underwent immunosuppression conversion. There was no significant difference in urine TGF-beta 1 values between treatment groups at either the start or the finish of the study (Table 6).

Table 6. Urine TGF-beta 1 measurements in patients with CAN according to immunosuppressive regimen

	MMF/ reduced dose CsA	Tacrolimus	CsA (no change)	P value (Kruskal- Wallis)
Start of study	37 (25-76) ^{1,2}	48 (0-97)	42 (27-66)	NS
At 6 months	41 (16-62)	114 (72-145)	47 (24-333)	NS

¹ expressed as a TGF-beta (pg/ml) to creatinine (mg/dl) ratio

² median (interquartile range)

Figure 17 is a plot of urine TGF-beta 1 concentration against 99mTcDTPA-GFR in patients with CAN at the start of the study. It can be seen that the correlation between the two measurements is poor, indicating that TGF-beta 1 expression is not a reliable marker of the chronicity and severity of CAN.

Figure 18 is a plot of urine TGF-beta 1 concentration against urine protein creatinine index (PCI) at the start of the study and shows that urine TGF-beta 1 excretion cannot be estimated reliably from a urine test that is used routinely in clinical practice.

In summary, these data suggest that TGF-beta 1 excretion in the urine is not a useful marker of CAN. A fundamental criticism of urine TGF-beta 1 as a marker of graft injury is that it may not reflect accurately the degree of activity of TGF-beta 1 *within* the graft. The latter can be studied using *in situ* hybridisation staining of transplant biopsy material but sampling error reduces the validity of this approach. Correction of urine TGF-beta 1 measurements based on the urine creatinine concentration may be flawed, as the latter depends on several factors other than the volume of urine output (such as diet, the size of the body creatinine pool and the extent of creatinine

Figure 15. Urine TGF-beta 1 measurements in CAN and control patients

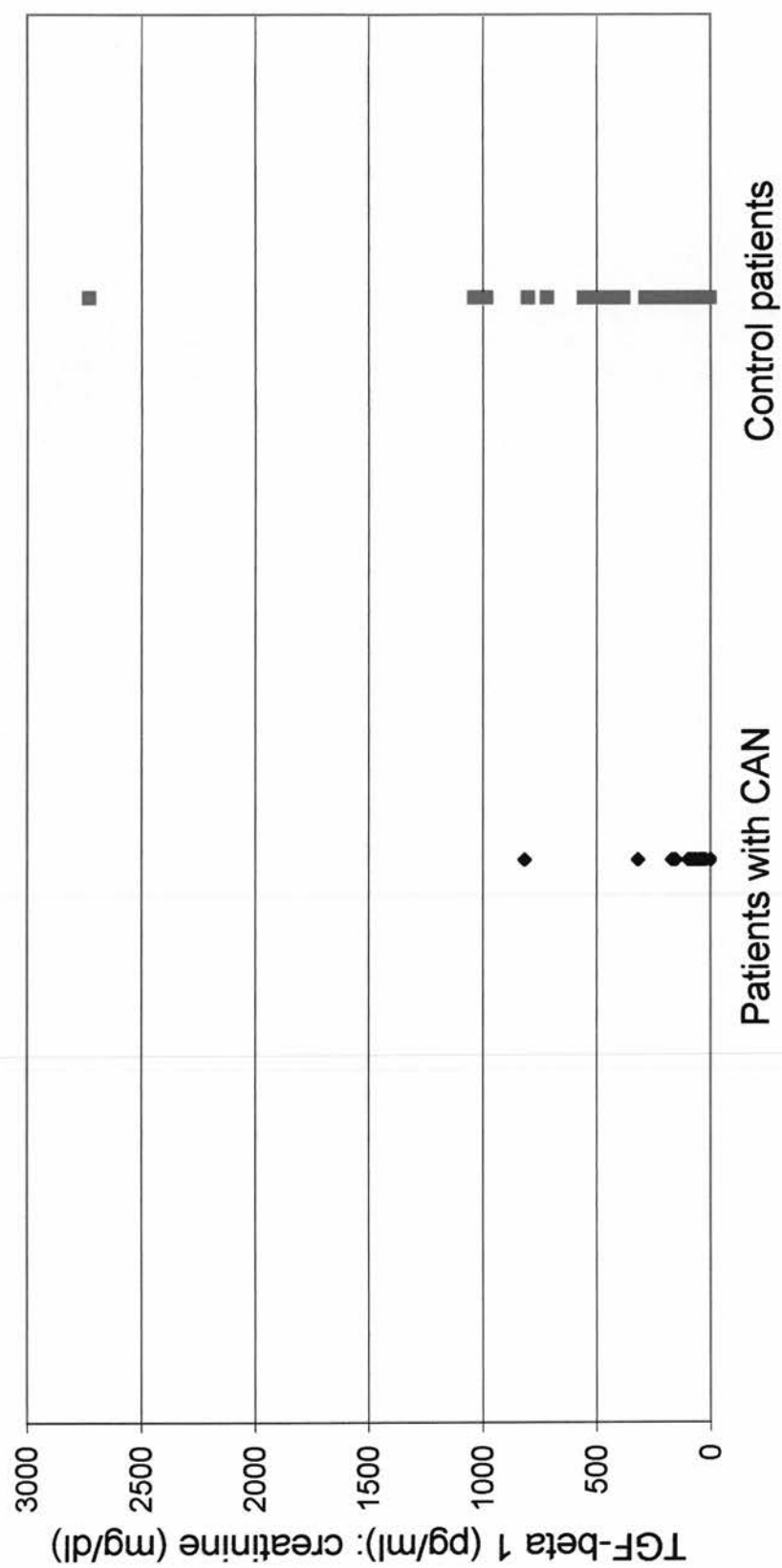


Figure 16. Urine TGF-beta 1 measurements in CAN patients during the 6 months study period

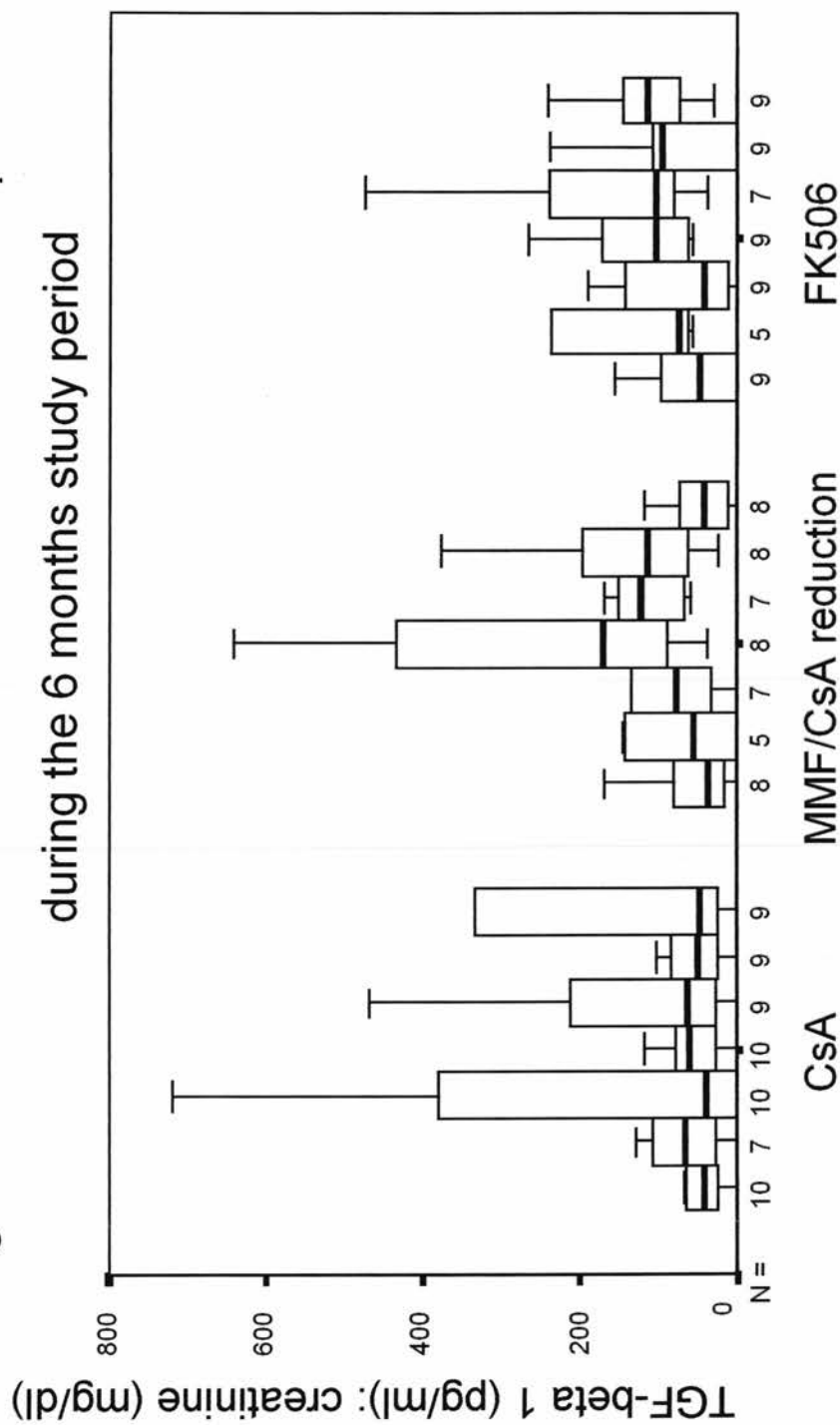


Figure 17. Urine TGF-beta 1 measurements in relation to GFR in
CAN patients at the start of the study

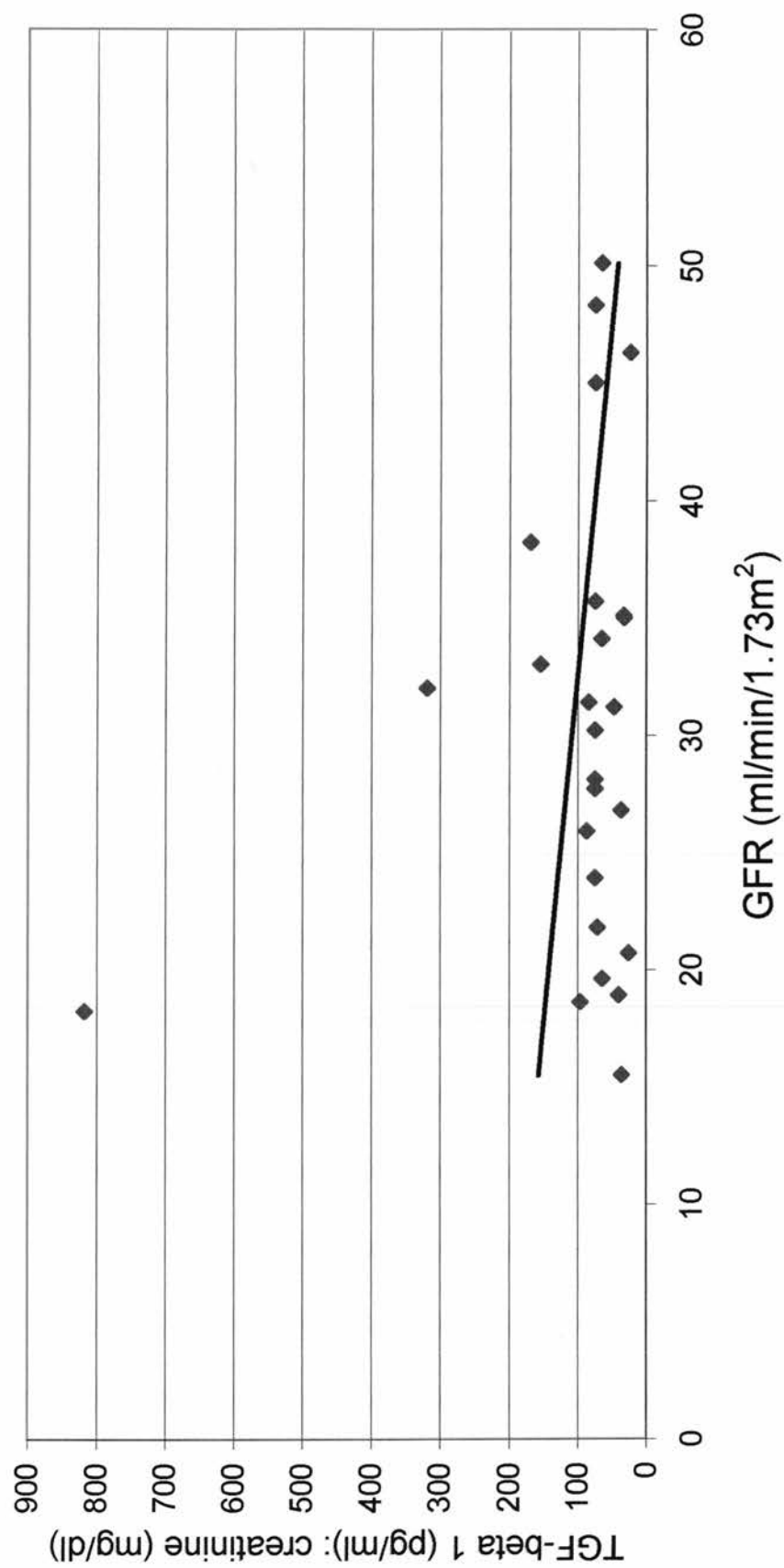
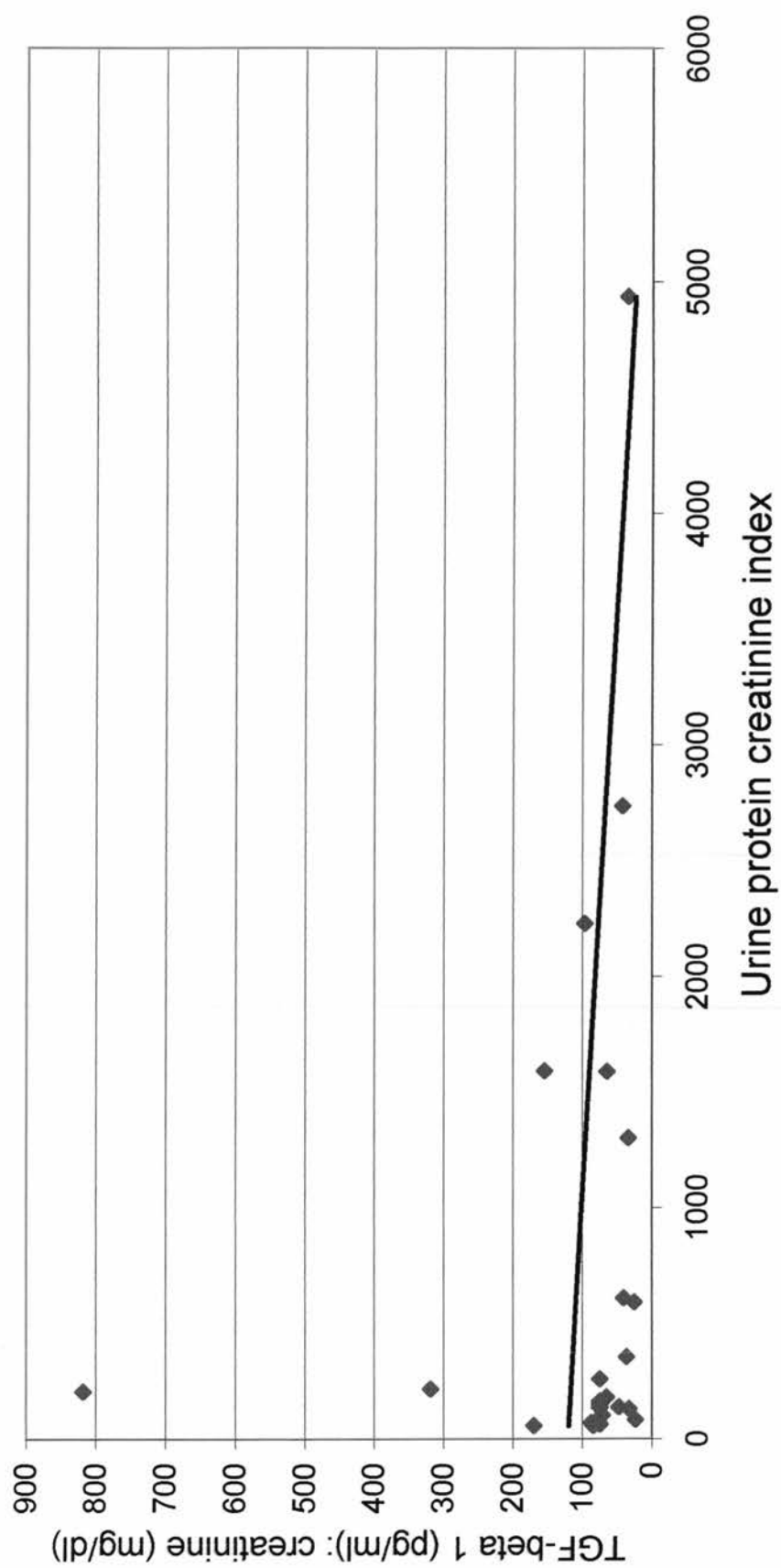


Figure 18. Urine TGF-beta 1 measurements in relation to protein creatinine index in CAN patients at the start of the study



creatinine secretion by the renal tubules). However, similar results to those presented above were obtained using *uncorrected* TGF-beta 1 values. It may be that a reduction in nephron mass influences TGF-beta 1 excretion, although in our study there was no correlation between urine TGF-beta 1 and GFR in patients with CAN. Prospective cohort studies are underway in Manchester and these may provide more information about patterns of TGF-beta 1 expression following renal transplantation.

6.3.2 NAG expression in urine

No patients had microbiological evidence of urosepsis at the time of sample collection. NAG excretion varied considerably between patients. Values for the CAN and control groups were not significantly different (Figure 19). There was no correlation between NAG excretion and GFR (Figure 20). In the CAN group, the change in NAG excretion over 6 months in the control and intervention groups was not significantly different (Figure 21), nor was there a significant difference between patients grouped according to the rate of decline in their transplant function over the same time period.

These cross-sectional and longitudinal data suggest that urine NAG excretion is not a specific marker for CAN in renal allograft recipients, nor is it a useful marker of an early response (as measured by change in GFR) to immunosuppression conversion therapy in such patients.

Modified studies that examine combinations of candidate non-invasive markers of incipient CAN may be contemplated in the future. Control groups need to be rigorously defined. As stated in Section 5.3.1, the addition of histopathological criteria to those specified in our study would reduce the likelihood of including

Figure 19. Urine NAG excretion in transplant recipients with and without CAN

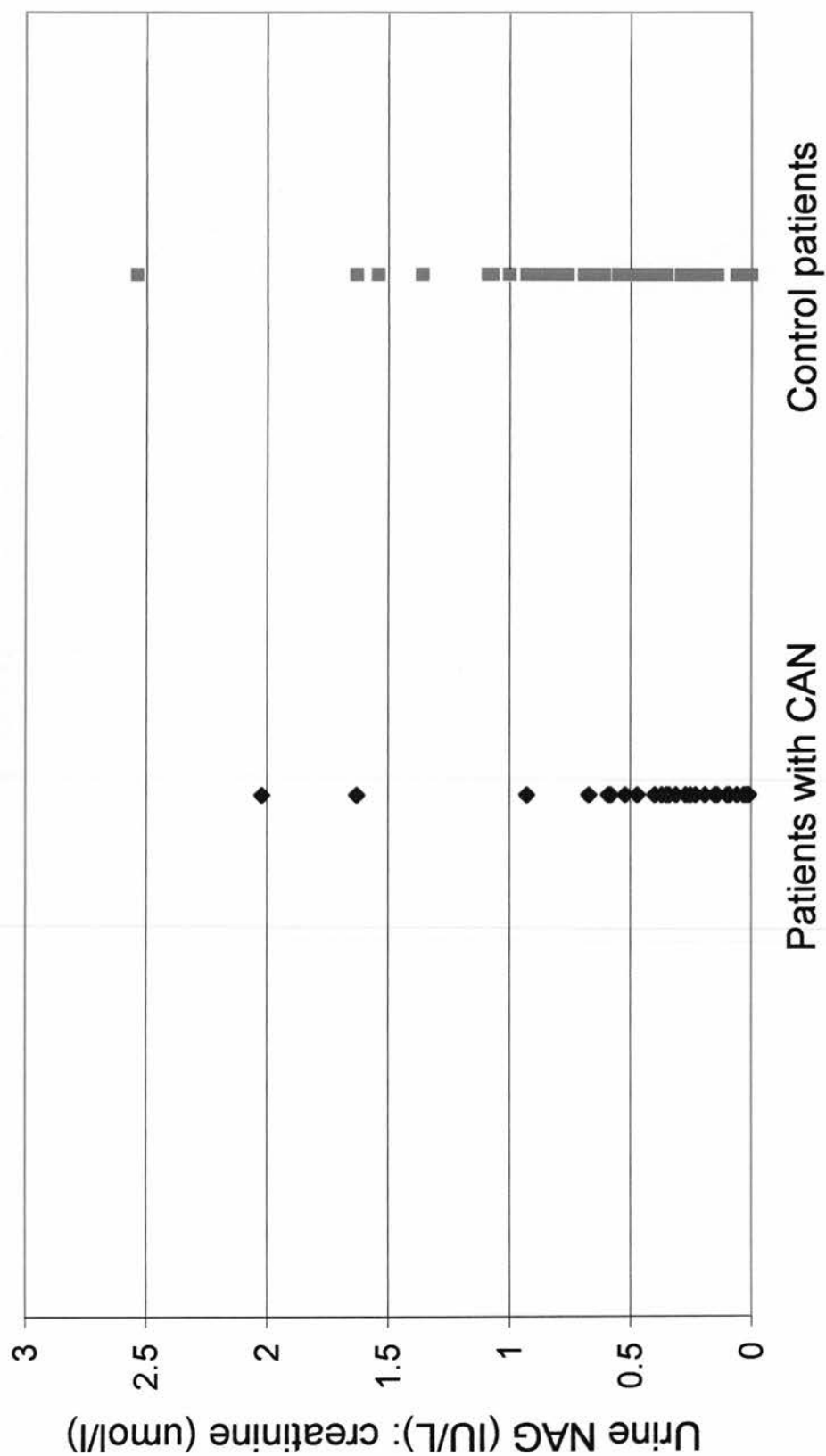
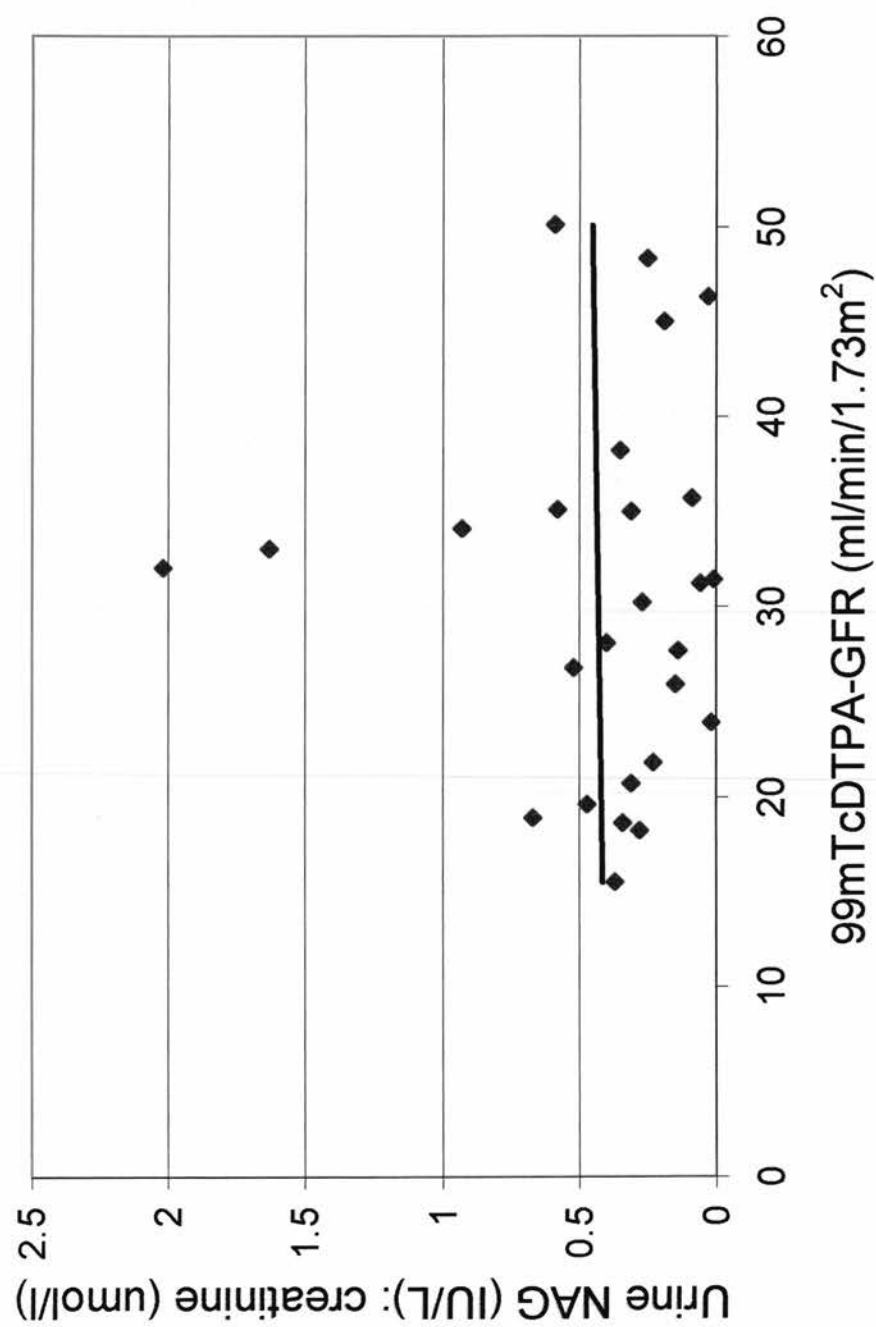


Figure 20. Urine NAG excretion in relation to GFR



patients with subclinical CAN as controls. *Proteomic analyses* may help to identify a family of discriminatory urine markers for inclusion in future non-invasive graft surveillance programmes. The term proteome refers to all the proteins expressed by a genome, and thus proteomics involves the identification of body proteins and the determination of their role in physiological and pathological functions. The ~30,000 genes defined by the Human Genome Project produce 300,000 to 1 million proteins when alternate splicing and post-translational modifications are taken into account. Whereas an individual's DNA genome remains essentially unchanged through life, the protein content of body cells changes continually as genes are turned on and off in response to variation in the cell environment. Proteins cannot be amplified like DNA, therefore less abundant sequences are more difficult to detect. In addition, enzymes, heat, light and aggressive mixing may alter the secondary and tertiary structure of proteins during the analytical process. Several major technologies are used in protein analysis, including 2-D polyacrylamide gel electrophoresis (2-D PAGE) for protein separation, mass spectrometry (combined with ionisation methods) for protein identification, post-translational analysis (enzymatic and antibody-binding methods to detect protein phosphorylation and glycosylation), and bioinformatics. *Protein microarray* technology has recently been developed and can be used for protein expression profiling. Many types of substances can be bound to protein microarrays including antibodies, receptors, ligands, nucleic acids and carbohydrates. Some surfaces have broad specificity while others are highly specific and bind only a few proteins from a complex sample. After the capture step, the array can be washed to reduce non-specific binding. When subjected to short bursts of laser light, the retained proteins become uncoupled from the array surface and can

then be analysed by laser ionisation time-of-flight mass spectrometry. One potential problem with proteomics in the context of early detection of CAN is that different urinary proteins (of glomerular or tubular source) may be expressed at different stages in the progression of the disease. Large, prospective longitudinal studies will be required.

6.3.3 GFR prediction formulae and measurement of serum cystatin C

GFR prediction formulae

The median value of the corrected 99mTcDTPA-GFR measurements in the population of renal transplant recipients with CAN was 28 (range 15-55) ml/min/1.73m². Bias and scatter values for the various GFR prediction formulae are shown in Table 7.

Table 7. Bias and scatter values for the GFR prediction formulae

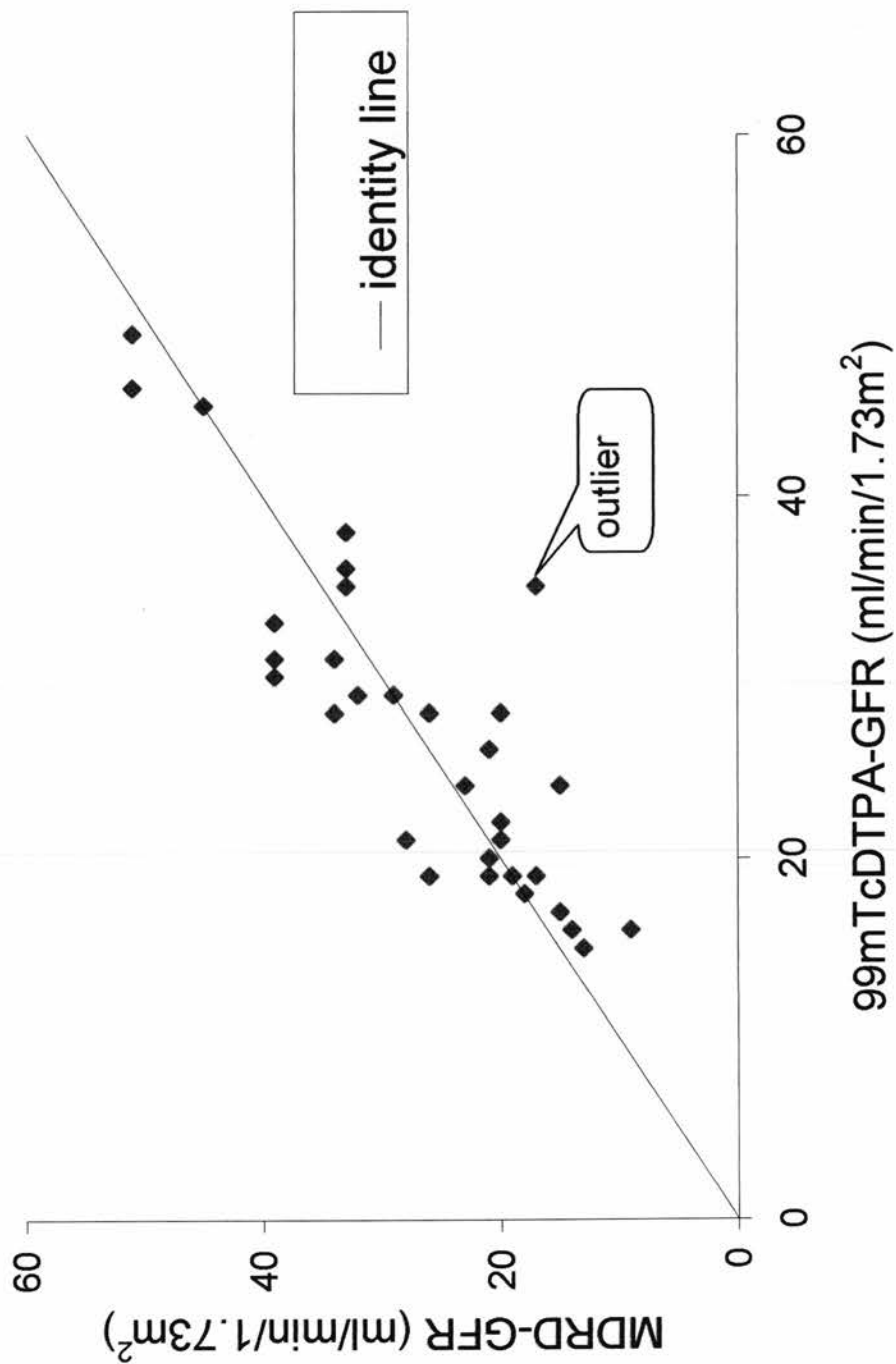
Formula	MDRD	Jelliffe	C & G	Walser	Nankivell	Bokenkamp
Required data ¹	A,G,R,C, U,Alb	A,G,C	A,G,C, W	A,G,C, W,H	G,Cr,U,W, H	Cystatin C
Bias (ml/min) ²	-1	+3	+7	-2	+7	+11
Scatter (ml/min) ³	3	5	6	4	10	11

¹ A - age, G - gender, R - race, Cr - serum creatinine, U - serum urea, Alb - serum albumin, W - weight, H - height

² median difference between calculated GFR and reference (99mTcDTPA-GFR)

³ median absolute difference between calculated GFR and reference (99mTcDTPA-GFR)

Figure 22. An identity plot for MDRD-GFR vs 99mTcDTPA-GFR



The MDRD formula gave the lowest bias and scatter (Figure 22). One patient was clearly an outlier, with an MDRD-GFR of only 17 ml/min/1.73m², compared to 35 ml/min/1.73m² measured by 99mTcDTPA clearance. The mean of his measured urea and creatinine clearances (from a 24-hour urine collection) was 34 ml/min/1.73m², suggesting that the MDRD-GFR estimate was inaccurate. The patient was a body-builder with an unusually large muscle mass.

Serum cystatin C and Cys-GFR

There was a strong correlation between 99mTcDTPA-GFR and the reciprocals of serum cystatin C and serum creatinine at 0 and 6 months (1/cystatin C vs 99mTcDTPA-GFR, $r = 0.83$ and 0.87 , $p < 0.001$; 1/creatinine vs 99mTcDTPA-GFR, $r = 0.78$ and 0.77 , $p < 0.001$; 1/creatinine vs 1/cystatin C, $r = 0.86$ and 0.87 , $p < 0.001$). Figure 23 illustrates graphically the relationship between 1/cystatin C and 99mTcDTPA-GFR values at the start of the study. There were two clear outliers, but repeat measurements at 6 months placed them closer to the regression line. Interestingly, the outlier for whom 1/cystatin C produced an *underestimate* of GFR was a body builder. He admitted to taking anabolic steroids, which may be significant in view of the recent findings of Risch and coworkers that glucocorticoids may increase serum cystatin C values (Risch *et al* 2001).

The calculated Cys-GFR values tended to exceed the corresponding 99mTcDTPA-GFR values, with a positive bias (median difference in values) of 10.9 ml/min/1.73m² and scatter (median absolute difference in values) of 11.1 ml/min/1.73m² (Figure 24). The Bokenkamp formula for Cys-GFR is based on serum cystatin C values that were obtained using a PETIA assay. Erlandsen *et al* reported a strong correlation between PENIA (the assay used in our study) and PETIA

Figure 23. Correlation between GFR and 1/cystatin C

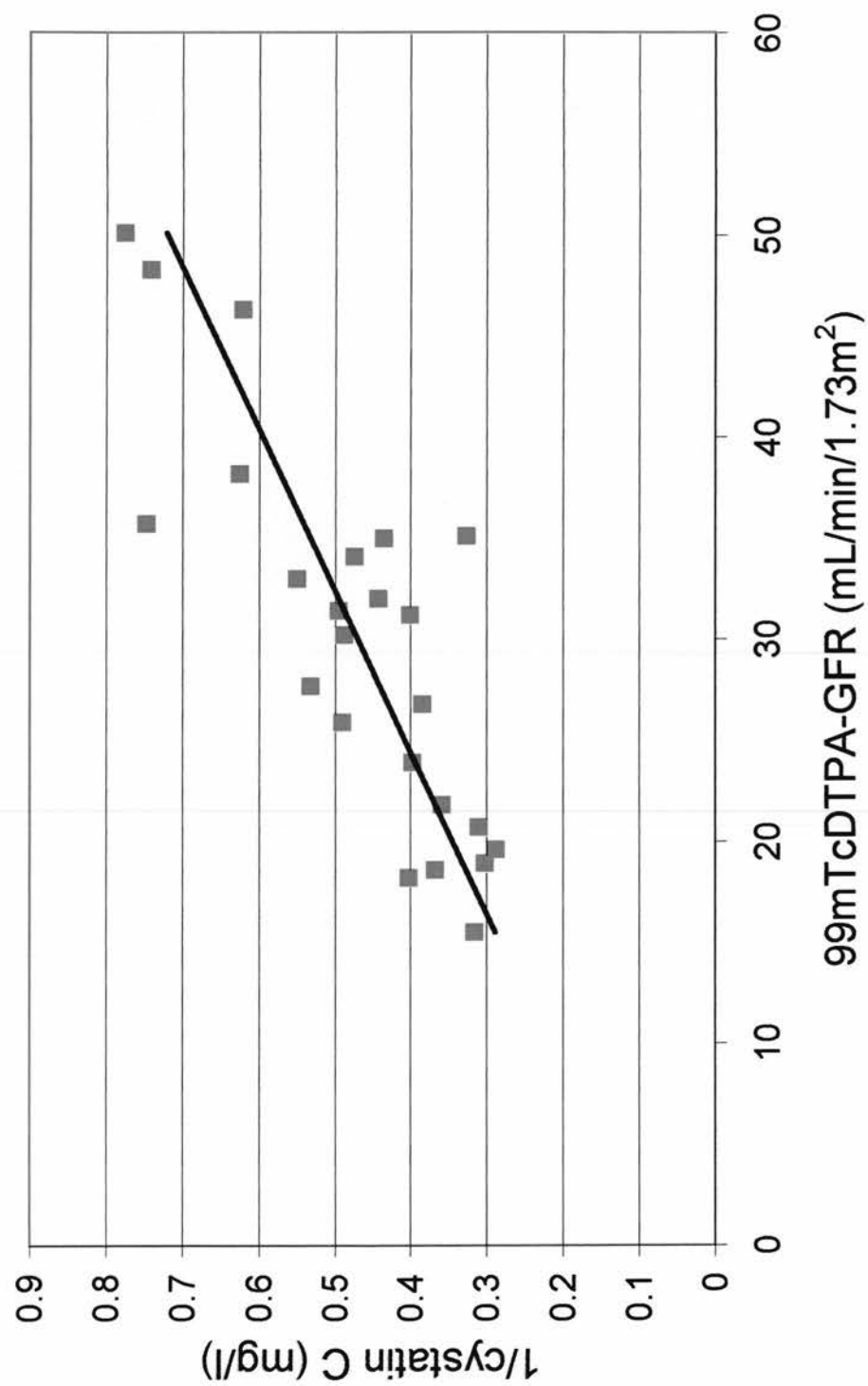
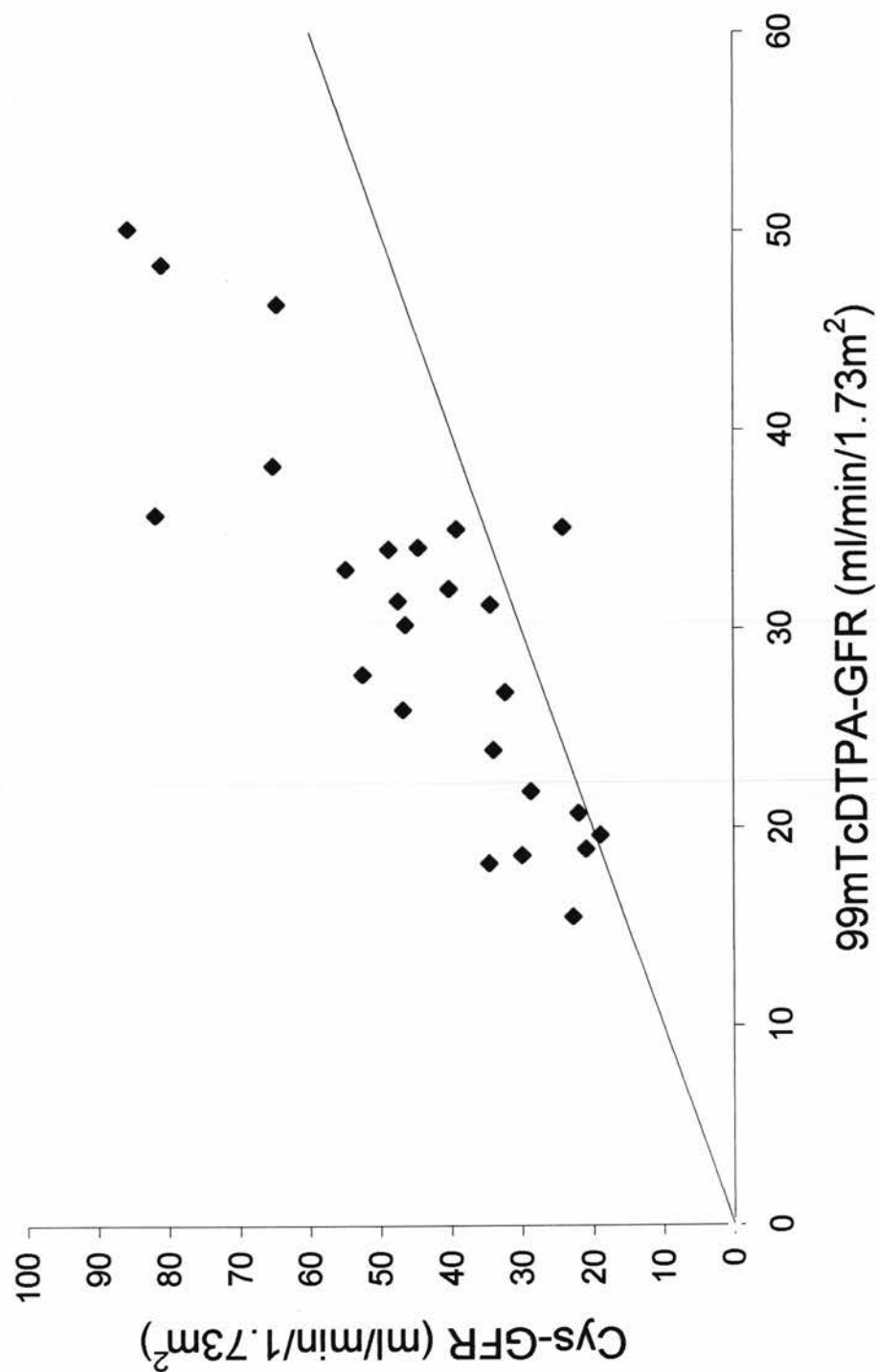


Figure 24. An identity plot for Cys-GFR vs 99mTcDTPA-GFR



(Erlandsen *et al* 1999). They have produced a regression equation to express the relationship of cystatin C values obtained using the two assays. We 'standardised' our PENIA-derived values using this equation but still found a large positive bias of approximately 7.5 ml/min/1.73m² for the Bokenkamp formula.

In summary, it can be concluded from the above studies of adult renal transplant recipients with chronic renal allograft dysfunction of wide-ranging severity that (1) the MDRD-GFR formula provides a valid estimate of renal clearance, and (2) serum cystatin C correlates more strongly with a 'gold standard' of GFR measurement (99mTcDTPA-GFR) than does serum creatinine. The MDRD-GFR formula is now available on a number of websites (including the Renal Association website at <http://www.renal.org/Resources/MDRD-GFRcalculator.htm>) and there have been discussions locally to facilitate the addition of MDRD-GFR to the laboratory reporting service. The most important advantage of serum cystatin C over serum creatinine is its greater sensitivity for detecting mild impairment of renal function. An earlier awareness of diminishing renal clearance increases the opportunity for effective therapeutic intervention. The PENIA assay is now fully automated and this may encourage the use of serum cystatin C as a measure of renal function in routine clinical practice.

Treatment of patients with established CAN – a
prospective study of immunosuppression
conversion

It has been suggested by several authors that the promising results achieved in pilot studies of immunosuppression conversion for CAN should be confirmed by larger, more rigorous studies that are prospective, randomised and controlled. Randomisation adds validity to a study by eliminating selection bias. The inclusion of a control group of patients whose immunosuppression remains unchanged and the use of gold-standard isotope techniques rather than serum creatinine to estimate renal clearance are also of crucial importance. The pattern of functional decline in CAN is non-linear, such that a phase of stability could be attributed falsely to a coincident change in treatment (Kasiske *et al* 1991). There may also be a confounding effect of study participation. For example, study participants may be managed by a relatively small number of senior research staff and receive more regular review than non-participants. For these reasons, it is clear that study findings are invalid unless control group data are available for comparison.

A reference method of measuring GFR is likely to provide much clearer information about renal function than serum creatinine, which can be misleading because of its poor sensitivity (as discussed in Chapter 3). In addition, non-renal factors (diet,

hydration status, patient medication and others) are known to have an effect on serum creatinine and the assays that are used in its measurement.

In response to the early reported experience with immunosuppression conversion for CAN and some encouraging results from a small study conducted in Liverpool, a team of senior surgeons and physicians from three regional transplant centres (Liverpool, Manchester and Leeds) met to formulate a larger, collaborative study.

7.1 Subjects and methods

Details of the study methodology are presented here in accordance with the revised CONSORT guidelines (Moher *et al* 2001).

7.1.1 Participating centres

The participating centres were St James's University Hospital (SJUH) in Leeds, the Royal Liverpool and Broadgreen University Hospitals (RLBUH) and Bradford St Luke's Hospital (BSLH). Potential study participants from the BSLH were identified, investigated and monitored by the research team at SJUH.

7.1.2 Investigators

The team at SJUH comprised a medical research fellow (Dr J Stoves), a research nurse (Sister H Ingles/ Mr J Carr) and a supervising senior consultant (Dr CG Newstead) with a specific interest in transplant medicine. The team in Liverpool included a surgical research registrar (Dr M Paraoan), a study coordinator (Mr G Owens) and a consultant transplant surgeon (Mr AQ Hammad). Laboratory work was performed in the Department of Renal Research at Manchester Royal Infirmary by Dr J Stoves under the auspices of Dr PEC Brenchley and his team of scientists (notably Dr S Williams, Dr B Coupes and Mr ID Read).

7.1.3 Planning of study and ethical approval

The study was planned in accordance with the Declaration of Helsinki (World Medical Association. Declaration of Helsinki 2001), Good Clinical Practice for

Trials on Medical Products in the European Union and the CONSORT guidelines (Moher *et al* 2001). Local Research Ethics Committee (LREC) approval of the study was obtained in both Liverpool (1998) and Leeds (1999) prior to its commencement.

7.1.4 Patient identification

The electronic records of renal transplant recipients receiving follow-up care in the participating units were reviewed systematically to identify patients with progressive allograft dysfunction, defined by a downward sloping 'reciprocal of serum creatinine versus time' (ROCT) plot over a minimum period of 6 months. Several reviews were performed during the course of the study. The suitability of patients for study entry was assessed in the clinic.

7.1.5 Entry criteria

The main inclusion and exclusion criteria for the study are summarised below:

Inclusion criteria

- 1) Above the age of 18 years
- 2) Receiving CsA-based immunosuppression
- 3) At least 6 months post-renal transplant, with no episodes of acute rejection during the preceding 3 months
- 4) Deteriorating renal function, in particular a sustained increase in serum creatinine for a minimum of 6 months
- 5) A normal renal transplant ultrasound and Doppler examination
- 6) Biopsy-proven CAN

Exclusion criteria

- 1) Previous treatment with tacrolimus, MMF or RPM
- 2) HIV, HBV or HCV infection
- 3) Serum creatinine of above 400 $\mu\text{mol/l}$
- 4) Pregnant or breast-feeding women
- 5) Female patients unwilling or unable to use approved contraception during the study and for 6 weeks following completion of the study
- 6) Presence of significant, uncontrolled concurrent infection
- 7) Participation in another clinical trial during the previous month.

7.1.6 Preliminary patient investigations

Patients who fulfilled the first four inclusion criteria and were otherwise eligible for participation in the study were referred for renal transplant ultrasonography and a colour flow Doppler assessment of the renal transplant vessels. The lead investigator proceeded to obtain a transplant biopsy specimen when the above investigations showed no abnormalities. Biopsy material was examined by a nominated histopathologist and classified according to the Banff 97 criteria (Racusen *et al* 1999).

7.1.7 Discussion and consent

Patients with biopsy-proven CAN patients were provided with a leaflet that contained important information regarding the background to the study, the rationale for intervention, the timetable of the study, profiles of the study medications

(tacrolimus and MMF) and issues of patient consent and confidentiality of study data.

7.1.8 Randomisation

Patients who consented to being included in the study were allocated randomly to one of the three treatment arms using a computer-generated sequence that was obtained from an independent source. Information regarding the randomised treatment was concealed in sequentially numbered sealed, opaque envelopes. These were opened in the presence of the patient (by JS at SJUH/ BSLH and GO at RLBUH) immediately after obtaining informed, written consent for participation in the study. All patients were allocated a unique numerical identification code based on the study centre to which they were attached and their place in the chronological order of randomisation within that centre. If a patient was removed from the study prematurely, his or her number was not reassigned to another patient. Both patient and physician were necessarily aware of the randomised treatment in all cases, but members of staff in the various clinical laboratories (including Medical Physics and Chemical Pathology) were blinded to this information. Prescriptions for new therapies were provided initially through the hospital pharmacy.

7.1.9 Treatment options

The randomised treatments were as follows: (A) MMF and reduced dose CsA; (B) tacrolimus in place of CsA; (C) 'no change' (continuation of a CsA-based immunosuppressive regimen). The protocol for each is summarised below.

A. MMF and reduced dose of CsA

- 1) MMF 500 mg bd added to regimen and azathioprine discontinued at enrolment
- 2) MMF increased to 750 mg bd and CsA dose reduced by 25% at the beginning of week 2
- 3) MMF increased to 750 mg bd and CsA dose adjusted to achieve a trough blood level of 75-100 ng/ml (monoclonal assay, Abbott Diagnostics, Delkenheim, Germany) at the beginning of week 3
- 4) In case of bone marrow suppression, MMF reduced as per the following regimen:
Peripheral blood white cell count $3-4 \times 10^9/\text{ml}$ – reduce dose to 750 mg bd
Peripheral blood white cell count $2-3 \times 10^9/\text{ml}$ – reduce dose to 500 mg bd
Peripheral blood white cell count $< 2 \times 10^9/\text{ml}$ – discontinue MMF for 10 days and restart at 500 mg bd if white cell count $> 4 \times 10^9/\text{ml}$
Neutropenia for more than 2 weeks - withdraw from study

B. Tacrolimus in place of CsA

- 1) CsA discontinued at enrolment and tacrolimus started at a dose of 0.1 mg/kg/day 12 hours after the last dose of CsA, or 24 hours if CsA trough blood level $> 300 \text{ ng/ml}$
- 2) Target tacrolimus trough blood level of 5-10 ng/ml (Tacro assay, Abbott Diagnostics, Delkenheim, Germany) during the study period

C. Control group

No change to treatment regimen, target CsA trough blood levels maintained as per unit protocol during the study period

Continuation of a CsA-based regimen has been regarded hitherto as a ‘best practice’ strategy for long-term immunosuppression. It was considered likely that any treatment disadvantage for the control group over a period of 6 months (the length of the study period) would be small.

7.1.10 Dosing and administration of additional medication

All treatments that were commenced during the study period were recorded, including dose and indication. ACE inhibitor and HMG-CoA reductase inhibitor (statin) medications were not added to patient treatment regimens (as per study protocol).

7.1.11 Safety assessment

Following enrolment, all patients were evaluated for adverse events. An adverse event was defined as *any* untoward medical occurrence (i.e. including events that did not necessarily have a causal relationship to trial medications). A clinical examination and laboratory safety tests were performed at each clinic visit.

7.1.12 Baseline investigations

Baseline investigations comprised the following: height, weight, blood pressure, GFR (measured by clearance of ^{99m}TcDTPA), full blood count (FBC), renal biochemistry, glucose, HbA1c (glycosylated haemoglobin), fasting lipids (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides), CsA trough blood level, urine protein creatinine index (PCI) and MSSU for microscopy, culture and

sensitivity. These investigations were repeated at varying intervals during the 6 months study period.

7.1.13 ^{99m}TcDTPA-GFR measurement

This comprised upper limb intravenous injection of a small volume of isotonic solution containing ^{99m} technetium of known radioactivity bound to DTPA and blood sampling from the contralateral limb at 2, 3 and 4 hours post-injection. The degree of residual radioactive signal present in the samples was plotted on a decay curve, from which a crude GFR was calculated and subsequently adjusted for body surface area using the Dubois formula (Dubois *et al* 1916). All GFR measurements were performed in the mid-morning to early afternoon period.

7.1.14 Subjective assessment of quality of life and medication side-effects using a validated questionnaire

Patients were asked to complete a validated, gender-specific questionnaire (Modified Transplant Symposium Occurrence and Symptom Distress Scale, Moons *et al* 1998) at the beginning and the end of the study after providing informed consent. The questionnaire comprised more than 40 questions, many of which focused on the recognised side effects of immunosuppressive therapy that occur in transplant recipients. Patients were able to grade both the frequency of occurrence and severity of specific symptoms. Intraindividual differences in questionnaire scores (obtained by subtraction of the final score from the initial score) are to be compared across treatment groups using Mann Whitney. Completion of the statistical analysis is the responsibility of the team in Liverpool.

7.1.15 Study schedule

Patients were given a study schedule containing dates for subsequent clinic visits. A letter confirming study entry was sent to the general practitioner. Patients were reviewed weekly for the first month, fortnightly for the second month and monthly thereafter. A second 99mTcDTPA-GFR measurement was performed at the end of the 6 months study period. Each patient's immunosuppressive regimen was reviewed following completion of the study.

7.1.16 Study outcome measures

Primary study outcome measure

The primary outcome measure was change in renal function. This was assessed by comparing 99mTcDTPA-GFR values at the start of the study and at its conclusion 6 months later, and also the slope of ROCT plots before and after treatment intervention (using creatinine data from 12 months pre-study to 12 months post-study entry).

Secondary study outcome measures

Hypertension and hypercholesterolaemia, two important side effects of immunosuppressive therapy, were selected as the secondary study outcome measures. Change in urine protein creatinine index (PCI, a measure of the degree of proteinuria) was also assessed.

7.1.17 Power calculation

A power calculation was performed to determine the number of patients that would be required for clinically relevant differences in outcome between treatment groups

to reach statistical significance. There are obvious problems with performing power calculations in this setting. Firstly, there is a variable rate of functional decline in some patients with CAN, such that slowing of disease progression may be independent of the therapeutic intervention. Secondly, the likely effect on allograft function of immunosuppression conversion is difficult to estimate because of insufficient preliminary data. Assuming a standard deviation (SD) of 4 ml/min/1.73m² per year for the annual reduction in GFR in patients with CAN, a study population of 48 patients would be required for a difference of 3 ml/min/1.73m² per year in the rate of deterioration between treatment groups to be statistically significant (p<0.05, power 80%).

7.1.18 Interim analysis

A statistician performed an interim analysis of the data after 36 patients had completed the study (three-quarters of the required number). The statistician was otherwise uninvolved with the study. There were two main reasons for not performing the analysis sooner (i.e. a bias towards collective ethics rather than individual ethics). Firstly, the differences in outcome between the three treatment groups were not expected to be large. Secondly, the study was of short duration and it was felt that the initiation of a more effective treatment (if this were to be proven) for a chronic pathology could be deferred without major consequences for individual patients.

The analysis was reviewed by senior clinicians who had been involved in the planning but not the execution of the study. There was no input from the study sponsors.

7.1.19 Statistical methods

We performed an 'on-study' analysis of patient data. The difference in slope of the pre- and post-intervention ROCT plots was calculated for each patient and an intergroup comparison of values was made using a Kruskal-Wallis test. It was not assumed that pre-study ($-12/12 - 0$) and post-study ($0 - 6/12$) slopes would have a common intercept at time zero. Intergroup comparisons of changes in GFR, cholesterol, triglycerides, systolic and diastolic blood pressure (SBP, DBP) between the start and the finish of the study were also made using a Kruskal-Wallis test. Analysis of covariance (ANCOVA) was performed to assess the relevance of the pre-study GFR to therapeutic response.

7.2 Results

The first study patient was enrolled in July 1999. It was decided in March 2001 that no patients would be recruited into the study after December 2001 because of a paucity of new cases in the three participating centres. The study was completed (last patient out) in June 2002. Forty-two patients entered the study, less than the target number derived from the power calculation. This was because of a slow take-on rate towards the end of the recruitment period.

Baseline data for the three study groups are summarised in Table 8. It is apparent that the groups were broadly similar. Age at transplantation and time post-transplant were higher in the MMF/ reduced dose CsA group and there was a larger proportion of female patients. Five patients were recipients of living donor grafts, two in the tacrolimus group and three in the control group. Four patients had pre-transplant diabetes mellitus, one in each of the intervention groups and two in the control group. There was no significant difference between groups in the distribution of pre-study ROCT linear regression slopes. HMG-CoA reductase inhibitors and ACE inhibitors were not added to any of the treatment regimens during the study period.

A patient flow chart for the study participants is shown in Figure 25. Two patients started dialysis treatment during the 6 months study period, one in each of the intervention groups. In both cases the initial GFR was below $20 \text{ ml/min/1.73m}^2$ and there was evidence of a rapid pre-study decline in graft function. An end-of-study GFR of 5 ml/min/1.73m^2 was assumed for both patients in the main GFR analysis. Similarly, two patients who failed to attend for an end-of-study GFR measurement

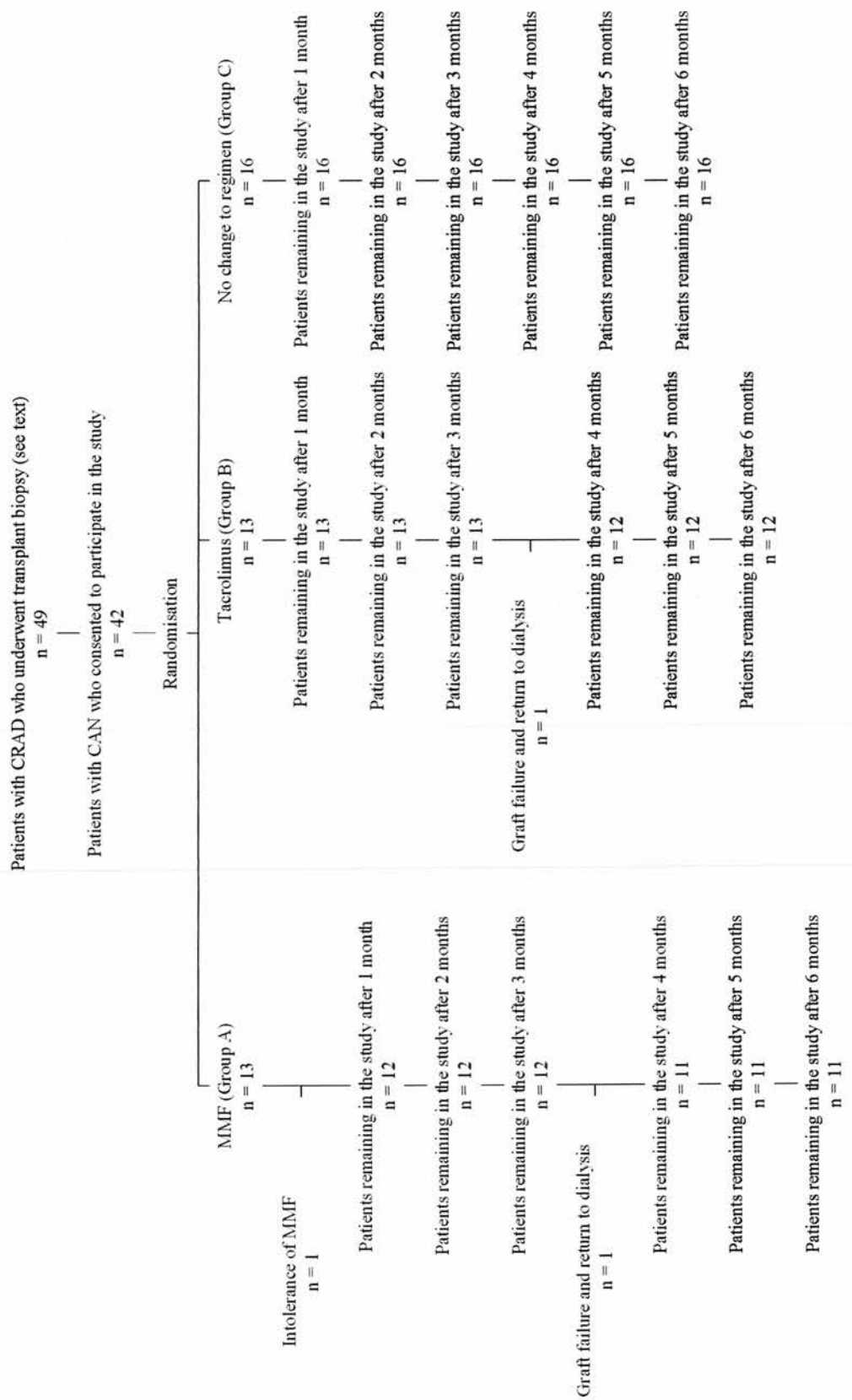
Table 8. Patient demographics by study group

	MMF/ CsA dose reduction	FK506 in place of CsA	Continuation of CsA
Age at transplant (years)	37 (27-47) ¹	26 (22-32)	30.5 (26.8-41.5)
Time since transplant (years)	8.7 (6.3-9)	7 (5-10)	4.6 (4.1-7.2)
Gender (male/ female)	6/7	10/3	12/4
Ethnicity (White/ Asian)	12/1	13/0	16/0
Donor age (years)	44 (22-50)	39 (19.5-43.5)	46.5 (42.8-53)
HLA mismatches (A/ B/ DR)	0.92/1.0/0.38	1.08/0.62/0.27	0.81/0.75/0.25
Previous immunosuppression (see footnote)			
PCA	9	9	8
PC	2	2	2
C	2	2	5
CA	0	0	1
CsA dose (mg/kg/day)	3.1 (3.0-4.9)	3.6 (3.2-4.3)	4.1 (3.5-4.7)
CsA trough level (C0)	146 (116-185)	142 (118-170)	160 (135-225)
Serum creatinine (umol/l)	206 (188-307)	216 (174-296)	235(171-328)
99mTc-DTPA GFR (ml/min/1.73m ²)	25.9 (19.7-35)	28(19-31.2)	23.9(19.1-35.1)
ACEI or AT2RA therapy	31%	46%	19%
Statin therapy	15%	15%	31%
Total cholesterol (mmol/l)	5 (4.5-5.8)	5.8 (5.4-6.1)	5.7 (4.9-6.3)
LDL cholesterol (mmol/l) ²	2.6 (2.4-2.9)	3.3 (2.8-3.6)	2.7 (2.7-3.2)
HDL cholesterol (mmol/l)	1.4 (1.3-1.7)	1.3 (1.3-1.6)	1.2 (1.0-1.4)
Triglycerides (mmol/l)	1.8 (1.0-2.0)	1.4 (1.0-3.0)	1.8 (1.3-2.2)
Systolic blood pressure (mmHg)	140 (130-150)	134 (126-150)	136 (128-143)
Diastolic blood pressure (mmHg)	85 (80-86)	83 (80-88)	81 (80-90)

P = prednisolone, C = cyclosporin, A = azathioprine

¹ Median value and interquartile range² Not performed in one of the three centres

Figure 25. A flow chart for study participants



(a control group patient and a patient who appeared to have responded well to a MMF/ reduced dose CsA regimen according to serum creatinine values) were not included in the GFR analysis. GFR was not measured in another control group patient because of her needle phobia.

One patient was intolerant of MMF and withdrew from the study after a few days of treatment. His data were disregarded as we wished to examine the 'on-study' effectiveness of treatments rather than analyse outcomes on an 'intention-to-treat' basis.

CsA dose was reduced by a median of 24% (interquartile range (IQR) 14%-27%) in the group receiving MMF, giving a median end-of-study CsA trough blood level of 99 (IQR 90-113) ng/ml. The median maintenance dose of MMF was 1.5 (IQR 1.5-2) g/day. The median end-of-study tacrolimus trough blood level in the group receiving tacrolimus was 7 (IQR 5-9) ng/ml. The median end-of-study CsA trough blood level for patients in the control group was 163 (IQR 145-215) ng/ml.

ROCT plots were constructed for each patient, taking creatinine data from 12 months before to 12 months after study entry. . During the 'run-in' period, serum creatinine was measured when patients attended for routine clinical review. The median number of creatinine values per patient over the 'run-in' period was 10 (interquartile range 7 to 12). A comparison of change in ROCT slope revealed a significant treatment advantage for patients in group A (Kruskal Wallis test, $p < 0.05$). Dotplots for post-treatment slope and change in slope are shown in Figure 26.

A separate analysis comparing the pre-study ROCT slope to the slope for the 3-12 months post-study period (i.e. excluding data collected in the first 2 months of the

study, by which time any beneficial effect of CsA dose reduction on renal transplant haemodynamics was likely to have occurred) also showed a difference between treatment groups, although this was not statistically significant (Figure 27, $p = 0.08$). Figure 28 is an identity plot to show the post-treatment ROCT slopes for the time periods 0-6 months and 3-12 months in the group of patients receiving MMF/ reduced dose CsA and standard dose CsA controls. Slopes were similar in control patients, whereas in the intervention group the 3-12 months slopes tended to be more negative than the corresponding 0-6 months slopes.

The analysis of GFR data (available in 37 patients) also showed a trend towards a treatment advantage for MMF/ reduced dose CsA (Figure 29, $p = 0.05$). The median increase in GFR after 6 months of follow-up for this group was 2.5 (IQR 0.3 – 9) ml/min/1.73m². The corresponding values for the tacrolimus and control groups were -0.6 (IQR -7.4 – 3.0) ml/min/1.73m² and -0.7 (IQR -1.4 – -0.1) ml/min/1.73m² respectively. Figure 30 shows the correlation between change in GFR and the slope of the ROCT plot between 0 and 6 months ($r = 0.61$, $p < 0.001$). An ANCOVA model including GFR at the start of the study (GFR0) as a covariate showed that GFR0 was a significant predictor of response to treatment ($p < 0.05$). To explore this further, patients with poor initial transplant function (GFR < 20 ml/min/1.73m²) were excluded from a repeat analysis. This showed a similar but more exaggerated trend toward a treatment advantage for MMF/ reduced dose CsA (Figure 31, $n = 27$, $p < 0.05$). The median increase in GFR in the MMF/ reduced CsA group was 3.3 ml/min/1.73m² compared to a median decrease of 0.7 ml/min/1.73m² in the control group.

Figure 27. Dotplots of change in ROCT slope for each study
group: pre-study vs 3-12 months

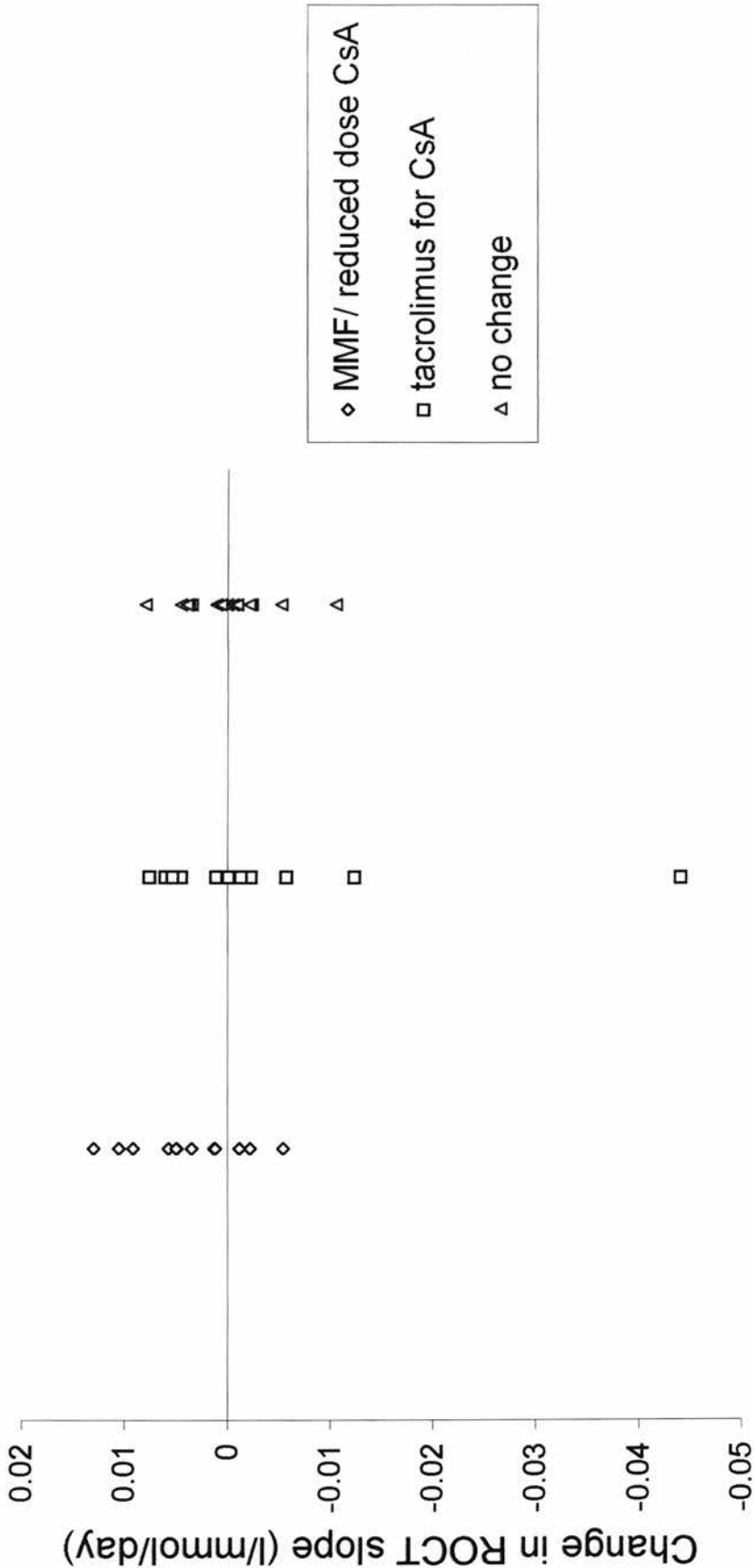


Figure 28. Change in ROCT slope for different phases of the study

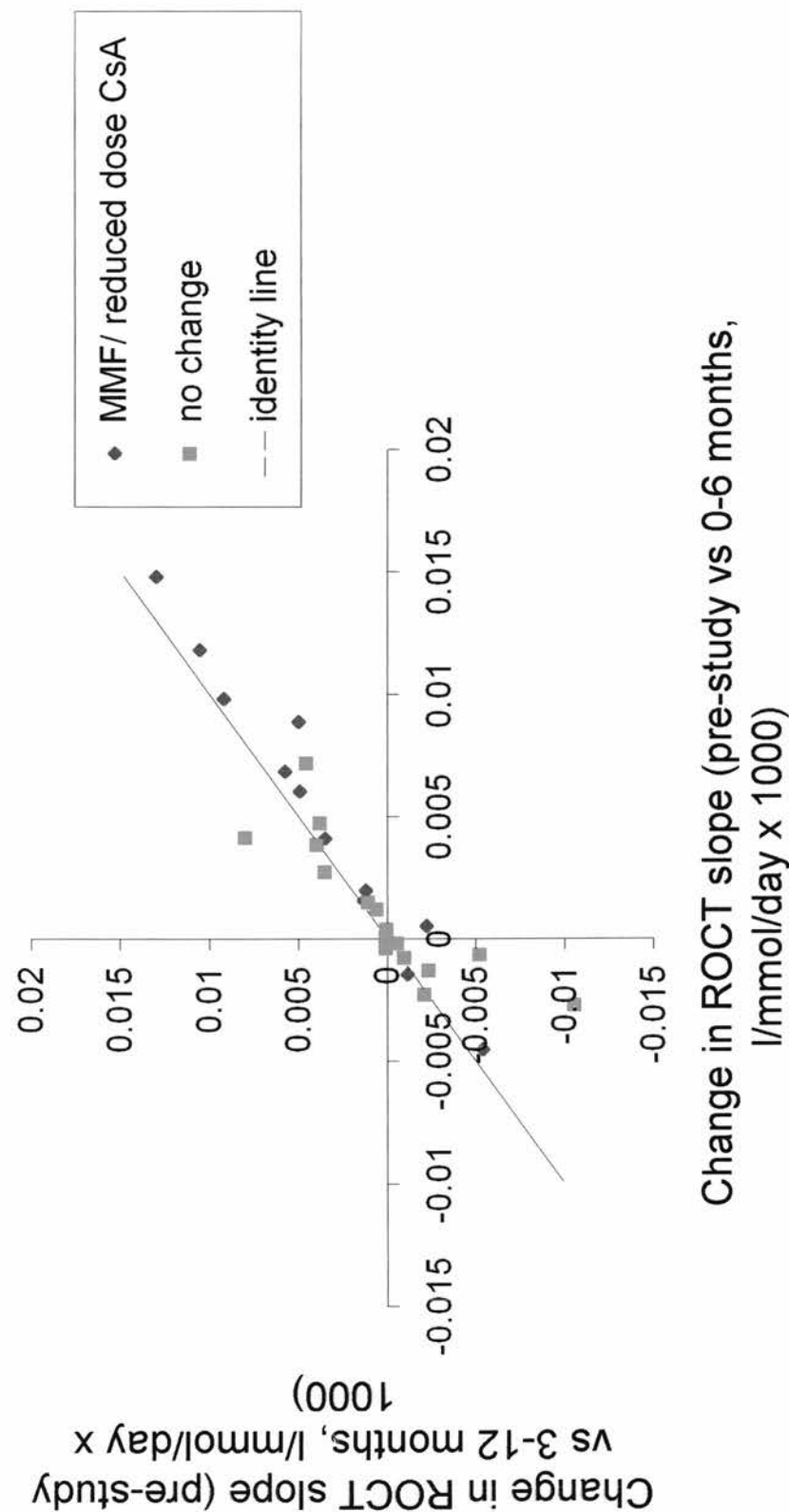


Figure 29. Dotplots of change in GFR for each study group
(all available data)

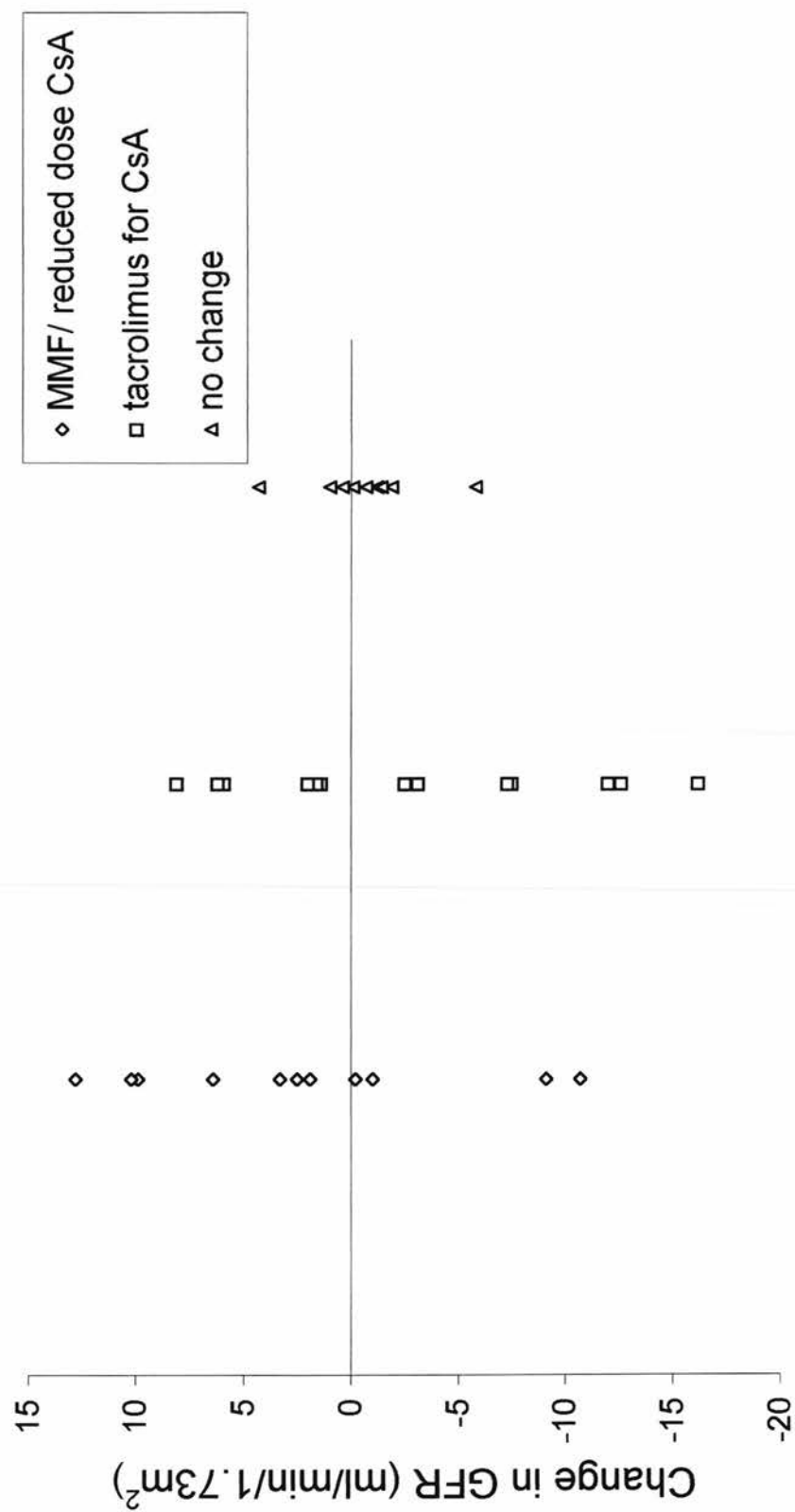


Figure 30. Correlation between the two methods of assessing change in transplant function

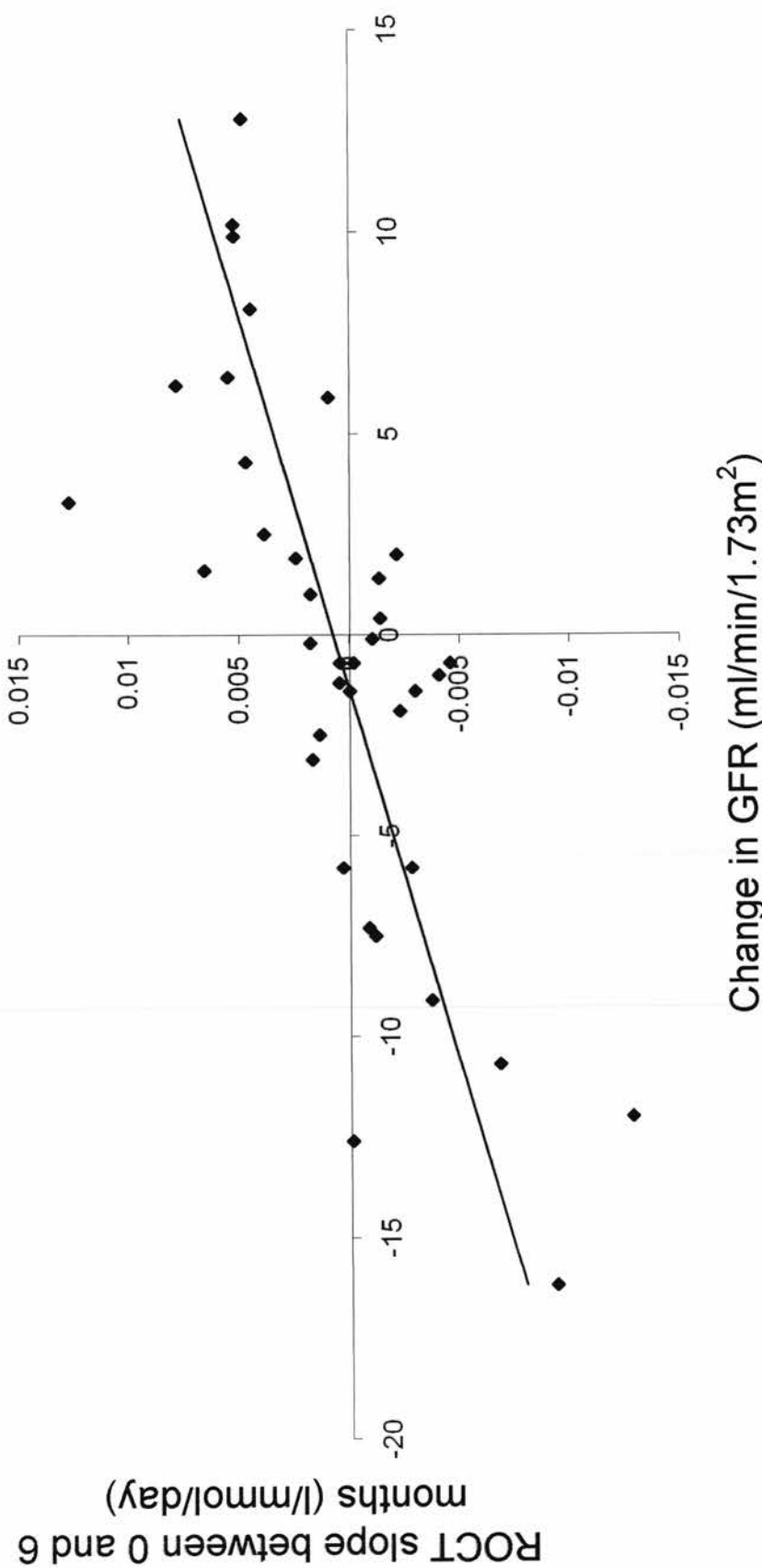


Table 9. Change in blood pressure, serum lipids and urinary protein excretion (pre-study minus post-study values) in the three treatment groups

Variable [<i>number with data</i>]	MMF/ CsA dose reduction	Tacrolimus in place of CsA	Continuation of CsA
Systolic blood pressure (mmHg) [41]	6 (-8 – 22) ¹	10 (-10 – 22)	5 (-10 – 20)
Diastolic blood pressure (mmHg) [41]	10 (1.5 – 15)	7 (0 – 11)	3 (-2 – 15)
Total cholesterol (mmol/l) [41]	0.3 (-0.7 – 0.9)	0.9 (0.1 – 1.4)	0.4 (-0.1 – 0.8)
Low density lipoprotein (LDL) cholesterol (mmol/l) [27]	-0.4 (-1 – 0.4)	0.6 (0.1 – 1)	0.1 (-0.2 – 0.2)
High density lipoprotein (HDL) cholesterol (mmol/l) [41]	0 (-0.1 – 0.3)	0.1 (0.0 – 0.2)	0 (-0.2 – 0.1)
Triglycerides (mmol/l) [41]	0.1 (-0.3 – 0.2)	0 (-0.3 – 0.8)	0.2 (-0.3 – 0.5)
Urine protein creatinine index (PCI) [27]	8 (-36 – 66)	-48 (-200 – 39)	-4 (-70 – 62)

¹ median (interquartile range)

The changes in fasting lipid profile, arterial blood pressure and proteinuria during the course of the study are summarised in Table 9. Blood pressure reduction was more substantial in the intervention groups but this did not reach statistical significance. There was also a non-significant trend towards an improvement in the serum lipid profile of patients receiving tacrolimus as compared to controls. There were no significant differences between treatment groups at the start and at the end of the

study period in terms of the number of prescribed antihypertensive agents. The change in urine PCI during the 6 months study period in the three treatment groups was not significantly different.

In the MMF/ reduced dose CsA group, gastro-intestinal disturbance occurred in six cases. This generally improved with MMF dose reduction. Two patients experienced insomnia. One patient required treatment for a recurring external ear infection. MMF dose was reduced in three patients because of progressive anaemia. One patient in the tacrolimus group developed progressive hair loss and her treatment was discontinued shortly after study completion. Another patient receiving tacrolimus required treatment for acne. Of the patients in the control group, one required surgical drainage of a perianal abscess, one developed acute gout and another required treatment for a urinary tract infection. None of the study patients developed acute rejection or *de novo* diabetes mellitus.

4.3 Discussion

This is the second prospective randomised controlled trial of immunosuppression conversion for CsA-treated renal transplant recipients with established CAN. The Creeping Creatinine Study examined the effect of introducing MMF followed by complete withdrawal of CsA and is unique in having a control arm (Dudley 2002). Renal function stabilised in 58% of patients receiving MMF in place of CsA ($n = 73$) as compared to 28% of controls ($n = 70$) during a follow-up period of 34 weeks. Three deaths occurred in the MMF group. There was no difference between groups in the incidence of acute rejection.

Our study provides evidence of a treatment advantage for MMF/ reduced dose CsA as compared to substitution of tacrolimus for CsA and standard dose CsA in patients with established CAN, at least in the short term. A reference method of GFR measurement ($^{99m}\text{TcDTPA}$ -GFR) was used to monitor change in transplant function in addition to more frequent serum creatinine measurements. $^{99m}\text{TcDTPA}$ -GFR was performed only twice (at the start of the study and 6 months later). ROCT plots gave more information about pre-study patterns of functional attrition and longer-term outcome (up to 12 months after study entry). Analyses of GFR and creatinine data gave similar results. It would have been preferable for the number of study participants to at least equal the power calculation estimate but this could not be achieved within the time limits of the study.

The apparent success of the above regimens could be explained simply in terms of the known effect of CsA on renal haemodynamics (Curtis *et al* 1986) and pro-fibrotic cytokine expression (Ghiggeri *et al* 1994; Shihab *et al* 1996). MMF is effective in

reversing some of the histopathological features of CAN in both renal and aortic allograft models of chronic rejection in the rat (Raisenen-Sokolowski *et al* 1994; Azuma *et al* 1995) and therapeutic concentrations of the drug inhibit fibroblast growth in cell culture studies (Zeier *et al* 2001). There is also some evidence that the use of MMF in place of AZA produces clinical benefit in terms of a reduced incidence of chronic renal allograft failure (Ojo *et al* 2000). Our comparison of pre-study and 3-12 months post-study slopes (attempting to separate the effects of MMF and CsA dose reduction, the latter producing an acute alteration in renal transplant haemodynamics) suggests that MMF may itself favourably affect long term outcome in CAN but this needs to be confirmed by a more extended period of follow-up of study participants.

It may be that the reported side effects of MMF therapy in the study participants were related to the degree of systemic exposure to the drug. Measurement of mycophenolic acid (MPA, the active metabolite of MMF) blood levels may have been informative in this regard.

The importance of intervening at a relatively early stage in the development of CAN is highlighted by two separate findings of this study. Firstly, baseline GFR (GFR₀) was found to be a significant predictor of outcome in an analysis of covariance (ANCOVA) model with GFR₀ as the covariate. Secondly, an analysis of change in GFR that excluded patients with a GFR of less than 20 ml/min/1.73m² revealed a more definite difference in outcome between the treatment groups. These findings emphasise the need for prompt detection of CAN, either by protocol biopsy or the use of more sensitive and specific tests of blood and urine (see Chapter 6).

The rate of change of GFR was reduced in some patients receiving tacrolimus, but the treatment response in the tacrolimus-treated group as a whole was not superior to controls. The characteristics of patients who appeared to respond well were not different to those of others in the group. There was a non-significant trend towards an improvement in the serum lipid profile of tacrolimus-treated patients as compared to controls.

In summary, this randomised, controlled comparative study of immunosuppression regimens in patients with established chronic allograft nephropathy provides evidence to support the use of MMF and low dose CsA in preference to a standard dose CsA-based regimen. More substantial reductions in CsA dose may produce an even better outcome, as this component of the regimen appears to have the greatest impact on graft function, at least in the short term. However, the increased risk of acute graft dysfunction associated with total CsA withdrawal needs to be considered (Stoves *et al* 2002a). Further studies to compare MMF-based regimens with newer immunosuppressive agents such as rapamycin may help to determine an optimal regimen for the prevention and treatment of CAN. Timely intervention depends on early detection of CAN. This is probably best achieved by protocol transplant biopsy or the development of valid non-invasive tests of renal allograft injury. Control of non-immunological factors such as blood pressure and serum lipids remains an important treatment goal.

Summary

Chronic allograft nephropathy (CAN) remains a major cause of late renal transplant loss despite substantial reductions in the incidence of early acute rejection. Renal transplant recipients with allograft failure secondary to CAN account for roughly 3% of all entrants to chronic dialysis programmes in the UK, increasing the demand placed on this limited resource. Allograft failure also has a considerable impact on patients awaiting call-up for transplantation, for example as many as 1 in every 6 patients transplanted in the UK in the year 2000 had previously received a renal transplant. It is therefore very important that the clinical management of renal transplant recipients (RTRs) should include anticipation, prevention, early detection and optimal treatment of CAN. The component studies of my thesis have addressed specific areas within each of these main divisions.

The notion of a screening programme for potential RTRs to reduce the prevalence of CAN through identification of those 'at risk' is a credible one. However, the inclusion in such a programme of genetic profiling to predict post-transplant TGF-beta 1 expression and measurement of lymphocyte sensitivity to CsA *in vitro* is not supported by the study data. Modified studies that examine larger populations and combinations of screening tests may be contemplated in the future. Control groups need to be rigorously defined. The addition of histopathological criteria to those

specified in our study would reduce the likelihood of including patients with subclinical CAN, although most UK transplant centres do not routinely perform transplant biopsies in patients whose graft function is stable.

It would also appear that the measurement of candidate urine markers for *incipient* CAN (TGF-beta 1 and the lysosomal enzyme NAG) is of little predictive or diagnostic value. Proteomic analyses may help to identify a family of discriminatory urine markers for inclusion in future non-invasive graft surveillance programmes. One potential problem with this approach is that different urinary proteins (of glomerular or tubular source) may be detected at different stages in the development and progression of CAN. Large, prospective longitudinal studies will be required.

The studies relating to measurement of serum cystatin C (a more sensitive marker of small reductions in GFR than serum creatinine) and calculation of GFR using the Levey (MDRD) prediction formula indicate that both are useful in the detection and/or quantification of early renal allograft dysfunction. An earlier awareness of diminishing renal clearance would increase the opportunity for effective therapeutic intervention.

We have also produced evidence that a more accurate quantification of CsA exposure may be important in reducing both the incidence of acute transplant rejection during the immediate post-transplant period (a well documented risk factor for the development of CAN) and the rate of chronic disease progression in established CAN. A large intra-individual variation in CsA exposure may indicate poor adherence to treatment regimens rather than variation in drug bioavailability. It is of course possible that calcineurin inhibitor therapy (in particular CsA) will not be used for maintenance immunosuppression in the future, as there are now alternative

agents such as rapamycin that are effective, cause less nephrotoxicity and have a more favourable pharmacokinetic profile (in particular a longer plasma elimination half-life).

In the study of immunosuppression conversion for RTRs with established CAN, a comparison of reciprocal of creatinine vs time (ROCT) slopes before and after intervention revealed a treatment advantage for patients whose immunosuppression was changed to MMF/ reduced dose CsA. Similar results were obtained in the GFR analysis. Exclusion of patients with an initial GFR < 20 ml/min/1.73m² from the analysis gave a result that was statistically and clinically more striking, indicating that immunosuppression conversion should be considered at an early stage in the development of CAN. The number of study participants was suboptimal, mainly because of slow recruitment in some of the participating centres. A more extended period of follow-up would have provided information about the longer-term safety and effectiveness of MMF in combination with reduced dose CsA for the treatment of CAN. Future research should include a comparison of MMF with other immunosuppressive therapies such as rapamycin, perhaps with more substantial concomitant reductions in CsA dose than were attempted in this study.

The research findings that are presented in this thesis have been disseminated at national and international meetings and in peer-reviewed journals (Stoves *et al* 2000, 2001a/b, 2002b/c/d/e, 2003, www.leeds.ac.uk/ykrf). Others may be encouraged to seek confirmation of our findings in larger prospective studies.

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